Effects of Aggregated Alpha-Synuclein on Cultured Neuronal Cells

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LOYOLA UNIVERSITY CHICAGO

EFFECTS OF AGGREGATED ALPHA-SYNUCLEIN ON CULTURED NEURONAL CELLS

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN CELL BIOLOGY, NEUROBIOLOGY, AND ANATOMY

BY

DAVID L. FREEMAN

CHICAGO, ILLINOIS

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ACKNOWLEDGEMENTS

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<tr>
<td>LBDs</td>
<td>Lewy Body Diseases</td>
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<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<td>PDD</td>
<td>Parkinson’s disease with dementia</td>
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<td>DLB</td>
<td>Dementia with Lewy Bodies</td>
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<td>MSA</td>
<td>Multiple System Atrophy</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>NLRP3</td>
<td>NOD-like Receptor family, Pyrin domain containing 3</td>
</tr>
<tr>
<td>MPP⁺</td>
<td>1-methyl-4-phenylpyridine</td>
</tr>
<tr>
<td>SNpc</td>
<td>Substantia Nigra pars compacta</td>
</tr>
<tr>
<td>L-Dopa</td>
<td>Levodopa (L-3,4-dihydroxyphenylalanine)</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>DBS</td>
<td>Deep Brain Stimulation</td>
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<tr>
<td>ADL</td>
<td>Activities of Daily Living</td>
</tr>
<tr>
<td>MAO-B</td>
<td>Monoamine Oxidase-B</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyltransferase</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
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<td>BD</td>
<td>Basal Ganglia</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>GPi</td>
<td>Globus Pallidus pars interna</td>
</tr>
<tr>
<td>GPe</td>
<td>Globus Pallidus pars externa</td>
</tr>
<tr>
<td>SNpr</td>
<td>Substantia Nigra pars reticulata</td>
</tr>
<tr>
<td>SNpc</td>
<td>Substantia Nigra pars compacta</td>
</tr>
<tr>
<td>STN</td>
<td>Subthalamic Nucleus</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>LB</td>
<td>Lewy Bodies</td>
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<tr>
<td>LN</td>
<td>Lewy Neurites</td>
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<tr>
<td>NF</td>
<td>Neurofilament</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>SPC</td>
<td>Spinal Cord</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted Laser Desorption/Ionization Time of Flight</td>
</tr>
<tr>
<td>NAC</td>
<td>Non-Aβ component</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide Association Studies</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Microscopy</td>
</tr>
<tr>
<td>AP</td>
<td>Action Potentials</td>
</tr>
<tr>
<td>Gal-3</td>
<td>Galectin-3</td>
</tr>
<tr>
<td>CRD</td>
<td>Carbohydrate Recognition Domain</td>
</tr>
<tr>
<td>IFA</td>
<td>Immunofluorescence Microscopy</td>
</tr>
<tr>
<td>chgal-3</td>
<td>mCherry labeled Galectin-3</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
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<tr>
<td>VSV-g</td>
<td>Vesicular Stomatitis Virus glycoprotein</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Dulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscope</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early Endosome Antigen 1</td>
</tr>
<tr>
<td>SIM</td>
<td>Structured Illumination Microscopy</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>H$_2$DCFDA</td>
<td>2',7'-dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>roGFP</td>
<td>Reduction Oxidation sensitive Green Fluorescent Protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>EMCCD</td>
<td>Electron-multiplying charge coupled device</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescent Intensity</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 Beta</td>
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</table>
CHAPTER ONE

INTRODUCTION

The protein alpha-synuclein (α-syn) can be found at the root of many overlapping neurodegenerative diseases. These conditions have been grouped together and termed, Lewy Body Diseases (LBDs). Included in this group of neurodegenerative diseases are: Parkinson’s disease (PD), Parkinson’s disease with dementia (PDD), dementia with Lewy Bodies (DLB) [1] and Multiple System Atrophy (MSA) [2, 3]. Parkinson’s disease is the most prominent and affects greater than 1% of the population over the age of 60, making it one of the most common neurodegenerative diseases [4-7]. Overall these diseases affect greater than 5 million people across the globe [2]. The incidence of PD alone has increased greatly in the past decade as the general population ages and average lifespan continues to increase. Unfortunately, the treatment for PD has not kept pace with the burgeoning patient population and neurologists have been left prescribing L-3,4-dihydroxyphenylalanine (L-DOPA), the dopamine precursor which has been the standard of care for PD since the 1960s [8].

The progressive and pathological accumulation of alpha-synuclein is the main characteristic linking all LBDs [2, 3, 9, 10]. Previously published studies have shown that
alpha-synuclein oligomers, or small aggregates, are toxic to cells in-vivo as well as in culture and that this toxicity can be passed from affected neurons to neighboring cells [11-14]. An understanding of the mechanism by which alpha-synuclein oligomers induce a cellular pathology that can be spread to surrounding cells will lead to a better understanding of the pathology underlying PD as well as a target for possible containment of the cellular damage [15-17].

Biochemical studies of alpha-synuclein have shown that when alpha-synuclein binds to lipid membranes it undergoes a structural transition to become an amphipathic alpha-helix [16, 18, 19]. This adoption of an alpha-helical shape also leads alpha-synuclein to induce membrane curvature, tubulation and disruption [20, 21]. These observations are remarkably similar to previous studies of adenovirus protein VI [22, 23]. Adenovirus, a non-enveloped virus, enters the cytoplasm of cells by disrupting the endocytic vesicles by utilizing a protein that the virus encodes for termed protein VI following internalization of the virus [24]. Protein VI also adopts an amphipathic alpha-helical conformation, the same structure and conformation of alpha-synuclein, which has been shown to be capable of disrupting and tubulating artificial liposomes [25] in a similar manner as alpha-synuclein. The vesicular disruption mediated by protein VI of the endocytic vesicle facilitates adenoviral infection, by allowing the virus access to the cytoplasm and nucleus. However, this rupture is sensed by the target cell as a “danger signal”, resulting in a cathepsin B dependent increase in cellular reactive oxygen species (ROS) [23] as well as NOD-like receptor family pyrin domain containing 3 (NLRP-3)
inflammasome activation [26, 27]. Alpha-synuclein oligomers have been shown to be internalized via the endocytic pathway [28, 29], the same pathway cells use to take up adenovirus. However, it remains unclear what occurs immediately following alpha-synuclein endocytosis. Alpha-synuclein is found in the cellular cytosol, so it must escape the endosome. The relationship between the escape of alpha-synuclein and the pathology induced by alpha-synuclein in vitro or PD pathology in vivo [17] is unknown. Given the structural similarities between alpha-synuclein and protein VI, we have hypothesized that the functions of these similar proteins would coincide.

Leading us to believe that alpha-synuclein would be able to generate a related rupture of intracellular vesicles and induce comparable downstream consequences such as ROS production in the affected cells. Indeed, from the data collected we were able to observe that addition of alpha-synuclein aggregates to neuronal cell lines induces the rupture of endocytic vesicles, specifically lysosomes, in neuronal cells that had taken up the protein aggregates. This lysosomal rupture induced a cathepsin B dependent increase in ROS in these neuronal cell lines. Notably, larger aggregates generated using alpha-synuclein with the E46K familial mutation did not induce the same degree of vesicle rupture or ROS induction. Alpha-synuclein aggregates were also shown to induce the secretion of exogenously labeled galectin-3. Grouped together, these experiments help to delineate a specific pathway by which cellular pathology may be introduced to cells during the propagation of alpha-synuclein and its associated pathology in PD. Gaining an understanding of the mechanisms that contribute to the toxicity seen following alpha-
synuclein aggregate endocytosis may help reveal valuable therapeutic targets for the millions of patients who suffer from the horrible symptoms of the LBDs. Downstream consequences of cellular exposure to aggregated alpha-synuclein are also elucidated in the data studies documented here as well as a possible novel mechanism of cell-to-cell transfer.

**Hypothesis**

The structure of alpha-synuclein endows the protein with the ability to perturb intracellular vesicles contributing to increased cellular stress and a propensity to propagate the cellular stress and downstream pathology.

**Specific Aim 1**

Demonstrate that alpha-synuclein can induce endosome/lysosome membrane rupture.

i) Define the aggregate species of alpha-synuclein that induces E/L rupture.

ii) Determine the relationship between alpha-synuclein aggregate addition and E/L rupture.

iii) Determine which intracellular vesicle/organelle is being ruptured.

iv) Determine if active cathepsins are released into the cytosol post E/L rupture.
Rationale

In its native state alpha-synuclein exists as an unfolded protein but it can adopt a variety of conformations and morphologies due to its lack of ordered structure [30, 31]. The discovery of missense mutations (A30P, E46K, A53T) [32-35] and structural mutations (E37K, E57K) in the alpha-synuclein protein led to intense debate about which aggregated form of alpha-synuclein is the most toxic. Previous studies have shown that alpha-synuclein can assemble into an oligomer, fibril or filament and that these ordered aggregations resemble the pathologic inclusions found in post-mortem tissue of PD patients [36-38]. While it is clear that the alpha-synuclein protein carries a heavy responsibility for the disease pathology the exact conformation that confers the greatest amount of toxicity remains unknown.

There has been evidence to show that extracellular alpha-synuclein is endocytosed by cells [39, 40]. However, there has been a lack of understanding in determining how alpha-synuclein mediates downstream cellular stresses after endocytosis. The course of endocytosed alpha-synuclein through the cell and any contributions the protein makes to the milieu of cellular stress is not clearly understood currently. One of the goals of this project aims to elucidate certain aspects of alpha-synuclein’s interaction with endosomal and lysosomal machinery utilizing fluorescent microscopy techniques. Following endocytosis it has been shown that alpha-synuclein can access the intracellular cytosol [16, 18, 41, 42]. The mechanism of release from an intra-organelle compartment to cytosolic compartment is an important transition that is not well understood.
We hypothesize that alpha-synuclein is operating similarly to adenovirus protein VI and rupturing the endocytic or lysosomal membrane. The rupture of the lysosomal membrane in particular could be disastrous for the cell, given the enzymatic content of that organelle.

Specific Aim 2

Demonstrate that alpha-synuclein can generate reactive oxygen species (ROS) production

i) Define the aggregate species of alpha-synuclein that most potently generates ROS.

ii) Determine the temporal relationship between alpha-synuclein aggregate addition and ROS generation.

iii) Determine involvement of lysosomal enzymes particularly cathepsins in relation to ROS generation.

Rationale

Oxidative stress has been well described in the pathology of aging and has been linked to neurodegeneration and LBDs [43-45]. We hypothesize that alpha-synuclein can generate ROS because of the pivotal role of mitochondria and alpha-synuclein in PD [46, 47]. Lysosomal rupture would surely release cathepsins which would activate the mitochondrial ROS pathway.
Specific Aim 3

Determine if alpha-synuclein is secreted through via a Galectin-3 mediated mechanism.

i) Determine the presence of alpha-synuclein in cell culture supernatant.

ii) Determine if Galectin-3 is present in cell culture supernatant.

iii) Determine if an exosome-like mechanism could be causing the secretion of alpha-synuclein.

Rationale

Neural grafts of fetal midbrain tissue transplanted into the striata of several PD patients demonstrated Lewy body pathology not only in the host tissue but in the newly grafted tissue as well [48-50]. This has led some to conclude that transfer of alpha-synuclein from cell to cell is occurring. Alpha-synuclein has been shown to leave cells through a variety of mechanisms and various forms of alpha-synuclein have been found in the cerebrospinal fluid (CSF), blood, and saliva [51-53]. This indicates that a certain percent of alpha-synuclein is in fact being secreted into the extracellular milieu. A recent publication by Thurston and colleagues has shown that one of the Galectin (Gal) family of proteins, Galectin-8, recognizes ruptured endosomes and targets the ruptured endosomes for autophagic degradation [54]. Galectin-3 has been shown to accumulate on damaged bacteria-containing vesicles but at this juncture the functional significance of its recruitment remains unknown. While it is clear that Galectins are β–galactoside binding lectins as aforementioned, and that these peptides are secreted into the extracellular
space. Their function in the extracellular space is also unclear. Because of this ambiguity and some small preliminary experiments performed in the lab we wish to examine the consequences of alpha-synuclein pathology on both the intracellular membranes and nuances of cellular stress as detailed in the previous two specific aims. We would be remiss if we did not address the possibility of galectin, a protein that can be found intracellular and extracellular playing a role in the mechanism of another protein that can also be found intracellularly and extracellularly, alpha-synuclein.
CHAPTER TWO
REVIEW OF LITERATURE

Parkinson’s disease and other Synucleinopathies

Parkinson’s disease (PD), Dementia with Lewy bodies (DLB) and Multiple System Atrophy (MSA) are all frequent disorders of an aging population and these diseases share a common characteristic: the progressive accumulation of alpha-synuclein in the central nervous system and the development of Lewy bodies [55]. Parkinson’s disease is undoubtedly the most well-known of these maladies with its first description being composed by the English physician Dr. James Parkinson in 1817, Essay on the Shaking Palsy [56]. Dr. Parkinson came to describe the condition as paralysis agitans and it was finally called Parkinson’s disease by the French physician Jean-Martin Charcot. PD affects between 100 to 200 per 100,000 people over the age of 40, with current estimates at greater than 1 million people affected in North America alone [5-7]. Parkinson disease is uncommon in people younger than 40, and the incidence of the disease increases rapidly over the age of 60, with a mean age at diagnosis of 70.5 years [4]. In fact, the first and most prominent risk factor for PD is age [57]. The macroscopic pathology of PD can be seen clearly; Lewy bodies, which are protein aggregates composed of alpha-synuclein as well as other proteins. These proteinaceous inclusions
are found in the substantia nigra pars compacta (SNpc) of PD patients and are the histopathological hallmark of these disorders [55]. However, the role of these aggregates and alpha-synuclein in PD pathology are not well understood. Gaining a better understanding of the cellular and molecular mechanisms responsible for the evolution of symptoms seen in PD will be critical for the development of new therapies designed to arrest disease progression. In PD, the role of excessive and misfolded alpha-synuclein has been best studied in the SNpc. In line with this data our lab has determined a relevant hypothesis and demonstrated some of the molecular mechanisms by which alpha-synuclein induces a cellular pathology that is spread from cell-to-cell.

Clinical Features of Parkinson’s disease

PD is the second most prevalent neurodegenerative disorder affecting over 6 million people worldwide [8]. The three classic motor signs that are seen in Parkinsonism are: bradykinesia, rigidity and resting tremor. By the time these motor symptoms have appeared it is believed that putamen dopamine uptake is decreased by greater than 35 percent [58]. The onset of motor symptoms is typically asymmetrical. Presence of two of the three cardinal signs combined with a response to the administration of L-Dopa is adequate for the clinical diagnosis of PD [59]. James Parkinson termed the disease paralysis agitans or “shaking palsy” and the resting tremor is the most visible cue to the observer of the neurodegeneration that underlies the clinical condition. Oscillating
movements of agonist and antagonist muscles create the tremor that is typically seen in PD. While the tremor is one of the first visible signs of the disease it is often most pronounced in the distal portions of the extremities, the fingers and hands are the usual culprits. As the disease progresses the tremor often decreases in severity while the rigidity and bradykinesia become more pronounced [59]. Rigidity can often be most clearly seen in the form of gait problems. PD gait consists of small shuffling steps with a lessened armswing and stooped posture [60]. Patients often have difficulty obtaining and maintaining their balance, which is part of the underlying etiology of the pronounced gait. Bradykinesia, which is defined as a slowness of movement can lead to akinesia or a complete lack of movement as PD progresses.

While the motor syndrome is what PD is infamous for, there are a range of non-motor symptoms as well. One of the most common symptoms that overlaps with DLB and Alzheimer’s disease (AD) is dementia. Dementia develops in 20-40% of PD cases[61]. Yet, the cognitive deficits are not exactly the same as what is typically seen in dementia cases, it usually includes executive impairment, episodic memory impairment, visuospatial dysfunction and impaired verbal fluency[62]. It has been suggested that there is an important interplay between the motor impairments commonly seen in PD and the cognitive deficits. Deep brain stimulation (DBS) does not ameliorate or alleviate the cognitive, psychiatric or verbal dysfunction seen in patients with PD.

*Current Treatment for Parkinson’s disease*
Currently no cure exists for PD and all that can be offered to patients is symptomatic management which can be done with close monitoring, precise medication adjustments, education, exercise and sometimes surgery. Once a diagnosis of PD has been made, the choice to start medical treatment depends on the severity of the symptoms that patient faces. Of paramount importance is the patient’s ability to perform activities of daily living (ADLs). There are currently 5 classifications of drug that may be used to treat the symptoms of PD: levodopa, dopamine agonists, anticholinergics, monoamine oxidase B (MAO-B) inhibitors, and catechol-O-methyltransferase (COMT) inhibitors [63]. All work at the neurotransmitter level in an attempt to replenish the missing dopamine. Levodopa is the most commonly used medication for the treatment of the motor symptoms of PD. It is often combined with carbidopa to prevent peripheral metabolism of levodopa and allow more levodopa to cross the blood brain barrier (BBB). In the United States formulations of carbidopa-levodopa are marketed under the name, Sinemet®. Correct dosing of Sinemet® is crucial, as the lowest dose possible should be used to control motor symptoms. The reasons for this are the dyskinesias associated with prolonged supplemental exposure to levodopa. Long-term exposure of five to ten years results in these abnormal involuntary movements for at least fifty percent of the patients taking levodopa [63, 64]. There are currently no treatments to halt or slow the progression of PD.

The most frequently performed surgery for the treatment of advanced PD is DBS [65]. Excessive STN excitation of the internal globus pallidus and excessive GP
inhibition of the thalamus are two of the downstream consequences of nigral/dopamine
deficiency. DBS addresses these inhibitory factors by electrically and reversibly
interfering with these inhibitory signals. These, in turn, cause reduced thalamocortical
activity, which is believed to mediate the symptoms of akinesia and rigidity.

_Parkinson’s disease and Basal Ganglia Circuitry_

While PD’s cardinal symptoms affect motor control these deficits originate from
the basal ganglia (BG). The BG is functionally as well as anatomically related set of
nuclei that include the caudate and putamen (together the caudate and putamen are called
the striatum), Globus Pallidus pars interna (GPi), Globus Pallidus pars externa (GPe),
substantia nigra pars reticulate (SNpr), substantia nigra pars compacta (SNpc), and the
Subthalamic Nucleus (STN). These nuclei are the principle components of the
extrapyramidal motor system which plays a role in the initiation of voluntary movements,
facilitation of some motion suppression and feedback from evolving motions [59]. The
majority of the afferent projections into the BG comes from the cerebral cortex and goes
to the striatum [66]. Efferent projections leave the BG from the GPi and SNpr and
activate the ventral anterior and ventral lateral thalamic nuclei (Figure #1). The ventral
lateral thalamic nuclei has a main function of movement execution and projects to the
primary motor cortex in the precentral gyrus of the frontal lobe, while the ventral anterior
thalamic nuclei has a main function of movement planning and projects to the premotor
and supplementary motor cortex of the frontal lobe [66].
The BG forms a complex network of circuits. However, there are two primary pathways through the BG circuitry: the “direct” and the “indirect” pathway [66]. In the “direct” pathway begins with putamen projections to the GPi as well as the SNpr, which are both output sources of the BG [66]. The “direct” pathway contains D1 dopamine receptors and co-expresses the peptides Substance P and Dynorphin. However, the main neurotransmitter used in the pathway is γ-aminobutyric acid (GABA), an inhibitory neurotransmitter and since the direct pathway is an inhibitory pathway when activated the reduction of inhibitory effects leads to a facilitation of movement. The “indirect” pathway links the putamen with the GPe and STN [66]. D2 dopaminergic receptors are used in the “indirect” pathway as well as the peptide enkephalin [59]. Activation of this pathway leads to a suppression of movement. Due to the presence of D1 and D2 dopamine receptors in the “direct and “indirect” pathway it is clear that dopamine can exert a dichotomous effect on each pathway: activating D1 receptors in the direct pathway and inhibiting D2 receptors in the indirect pathway. Yet, these two contrasting pathways when activated by dopamine come to facilitate movement.
A progressive loss of dopamine has been well established as one of the key outcomes of PD (Figure #2). This deficiency reduces inhibition of the “indirect” pathway and diminishes excitation of the “direct” pathway which creates an overall increase of
inhibition in the motor system (Figure #2) [59]. These “direct” and “indirect” pathway models provide a good framework for PD, but unfortunately they do not provide any insight into the etiology and pathophysiology of PD.

Figure #2: Basal Ganglia circuitry in Parkinson’s disease

Figure #2: Basal Ganglia Circuitry in Parkinson’s disease. Image by Mikael Haggstrom from Wikipedia commons used under Creative Commons Share 3.0.
Selective Neuronal Vulnerability in Parkinson’s disease

The underlying characteristics of these specific SNpc neurons that allow them to be specifically vulnerable remains a fiercely contested question. Many of the risk factors as well as environmental factors involved in the etiology of PD are either ubiquitously expressed or systemically exposed. What is distinctive about the cellular milieu and environment created by the SNpc that makes the neurons in that area so vulnerable? SNpc neurons are in fact physiologically different from the vast majority of other neurons present in the brain. Their distinction from other neurons originates from the SNpc’s ability to autonomously generate action potentials (AP) without any synaptic input [67]. APs are generated around 2-4 hertz and this rhythmic pacemaking activity is thought to help maintain high intracellular levels of dopamine [68]. This is in stark contrast to the electrophysiology driving most neurons, which consists of monovalent cation channels. SNpc neurons allow calcium to enter the cytoplasm through L-type calcium channels that have a distinct Ca,1.3 poreforming subunit encoded by Cacna1d [69, 70]. The Ca,1.3 subunit alters normal L-type calcium channel behavior by forcing the channel open during hyperpolarization which helps the cell reach threshold potential [69].

The sustained engagement of Cav1.3 calcium channels during pacemaking comes at an obvious metabolic cost to SNpc neurons. Because of its
involvement in cellular processes ranging from the regulation of enzyme activity to programmed cell death, calcium is under very tight homeostatic control, with a cytosolic set point near 100 nM; that is 10,000 times lower than the concentration of calcium in the extracellular space. Calcium entering neurons is rapidly sequestered into the sarcoplasmic reticulum or pumped back across the steep plasma membrane concentration gradient; this process requires energy stored in ATP or in ion gradients that are maintained with ATP-dependent pumps (Figure 3). In most neurons, calcium channel opening is a rare event, occurring primarily during very brief action potentials. [71]

The presence of this Cav1.3 subunit makes SNpC neurons substantially more vulnerable to any other stress, particularly any additional metabolic stressors as the metabolic machinery must overly exert itself in order to maintain calcium homeostasis.
Figure # 3: Calcium transport in SNpC neurons. [71]

Parkinson’s disease and alpha-synuclein
PD is characterized by a progressive loss of specific dopaminergic neurons in the BG as well as the presence of Lewy bodies (LB) and Lewy neurites (LN) [8]. LBs are intracytoplasmic aggregations of the protein alpha-synuclein (Figure #4) which can also be found intra-axonally and are called Lewy neurites (LN) [72-75]. These LBs and LNs were found to localize with alpha-synuclein staining in both sporadic and familial cases of PD [73, 75]. In many cases, LNs are found to be present without LBs, suggesting that LN formation may occur prior to LB formation [55]. DLB and MSA are two other synucleinopathies that have been found to contain neuronal as well as glial cytoplasmic inclusions that contain alpha-synuclein immunoreactivity [55]. Alpha-synuclein immunoreactivity is not visible in neuronal cells under normal conditions [55]. Friederich H. Lewy was the first to describe strange cytoplasmic inclusions found in the nucleus basalis of Meynert and the dorsal vagal nucleus in PD patients over one hundred years ago [76].

LBs composition has been a key area of research and it was uncovered that they are composed of filamentous structures [77, 78]. These filamentous structures resemble neurofilament (NF) but are thicker with a complete lack of side-arms [55]. LBs can be found through the central nervous system (CNS): hypothalamus, nucleus basalis of Meynert, substantia nigra, locus ceruleus, dorsal raphe nucleus, dorsal vagal nucleus, intermediolateral nucleus of the spinal cord (SPC) [79, 80]. Yet documented instances of LBs are not limited to the CNS, with recent papers showing the presence of LBs in the
sympathetic ganglia, the enteric nervous system, adrenal medulla, and the cardiac plexus [10, 55, 81-83]. This distribution of LBs to a myriad of different places in the nervous system underscores the variety of symptoms that can be present in a PD patient as well as the dramatic variability seen in the symptoms of the other LBDs.

LB’s linked alpha-synuclein to PD. However, alpha-synuclein is a multifunctional protein that has been linked to numerous cellular processes [84]. Another integral link between alpha-synuclein and PD was unearthed in 1997 when Polymeropoulos and colleagues found a point mutation (A53T) in alpha-synuclein that was linked to an early-onset autosomal dominant genetic form of PD [32]. While the mutation was only present in a small population of Italian and Greek families it had a high penetrance leading to the significance of the finding [32]. In the same year, full length alpha-synuclein was found to be the major component in the proteinaceous aggregates known as LBs found in autopsy tissue from sporadic PD patients [73, 75]. Two other point mutations have since been found (A30P and E46K) further strengthening the link between alpha-synuclein and PD [85, 86]. Gene dosing of the alpha-synuclein gene has been shown to be associated with PD in rare duplication or multiplication events [87, 88]. The genetic link between alpha-synuclein and PD is clear for familial PD and recently genome-wide association studies (GWAS) have shown that sequence variants in SNCA, the gene the encodes alpha-synuclein, are strongly associated with the sporadic form of PD [89, 90]. Put together these studies provide strong evidence that the protein alpha-synuclein plays a key role in the pathophysiology of PD.

**Alpha-synuclein structure**

Alpha-synuclein is 140 amino acids long, hydrophilic affinity for membranes, natively unfolded protein that is present in large numbers of adult human neurons. It was
originally found in Torpedo *californica* [91]. Being found to localize in two distinct cellular locations: the nuclear envelope and the presynapse, the protein was given the moniker “synuclein” [8, 84]. While alpha-synuclein is found only in mammalian species with a significant amount of conservation between species sequences, synucleinopathy only occurs in humans [84]. There exists a synuclein family of proteins: alpha, beta and gamma and these proteins share a common amino terminal sequence [8, 84]. The alpha-synuclein gene maps to chromosome 4q21.3-q22 [92, 93]. Alternative splicing generates three separate alpha-synuclein isoforms for humans: 140 amino acids, 126 amino acids and 112 amino acids [16]. The 140 amino acid isoform is the full length transcript and the most abundant [94]. The 126 amino acid isoform is generated due to an in-frame deletion of exon 3, while the 112 amino acid variant is caused by a in-frame deletion of exon 5 [95]. Alpha-synuclein is so abundant that it has been estimated to make up one percent of the total protein in soluble cytosolic brain fractions [96]. Knockout of the alpha-synuclein gene generates viable offspring which have small increases in dopamine release and a reordering of the vesicular synaptic pool [97, 98]. It is believed that this is a result of an alteration in synaptic vesicle exocytosis or endocytosis because either could lead to an upregulation in neurotransmisstion. In-vivo overexpression of alpha-synuclein has been correlated with a small decrease in secretion of neurotransmitter, most notably dopamine [39, 99]. In-vitro and in-vivo evidence has indicated that both reduction as well as overexpression of alpha-synuclein can induce neurodegeneration [100].

Overexpression of alpha-synuclein leads to an increase in protein aggregates [9].
The molecular mass of alpha-synuclein, as predicted by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) is 14,463 very close to the mass predicted from the amino acid sequence. However its large Stokes radius (34 Å) indicates that the native protein is in an elongated form [84]. It has been well established that alpha-synuclein has a natively unfolded structure. This unfolded structure is a result of three distinct regions of the synuclein transcript: 1) N-terminal region, 2) central region and 3) C-terminal region (Figure #5). Residues 1-60 constitute the N-terminal region which includes a seven time imperfectly recurring 11 residue sequence that has a highly conserved hexamer (KTKEGV) motif [21, 84, 95, 101]. The highly repetitive nature of this sequence gives rise to the amphipathic alpha-helical structure of alpha-synuclein. It is this structure, which is akin to the lipid-binding domain of apolipoproteins that allows substantial interaction with lipid membranes [102]. It is worth noting that within this N-terminal region lie the three specific mutations that give rise to some cases of familial PD (A30P, E46K, A54T). The central region, consists of residues 61-95 and within that stretch of amino acids is the non-Aβ component (NAC) region [93, 103, 104]. The NAC sequence is a highly hydrophobic region which is prone to aggregation and in fact necessary for alpha-synuclein aggregation [103]. Residues 96-140 compromise the C-terminal region which contains cation binding sites and is characterized by a high amount of acidic proline residues [8, 84].
Alpha-synuclein Lipid and Membrane Interaction

When alpha-synuclein is in its monomeric state it exists in a flux between being free in the cytoplasm and being bound to lipid membranes. While unbound in aqueous solution alpha-synuclein is mostly a random coil but when exposed to lipid membranes it quickly adopts an alpha-helical secondary structure [105]. The amino acid sequence which makes up the N-terminal region as well as the localization of alpha-synuclein at the presynapse indicates that it is a protein built to play a role in the membrane. Specifically, the alignment of the nonpolar hydrophobic residues into the lipid bilayer and the polar hydrophic residues in the opposite direction toward the aqueous cytoplasm allows alpha-synuclein a partial insertion into the lipid bilayer without the traditional transmembrane domain. This partial insertion is also secured in the lipid bilayer from lysine residues that are laterally aligned and allow further interaction with the negatively
charged phospholipid head groups [18, 31, 106] (Figure #6). Whilst binding to the membrane, the N-terminal portion of alpha-synuclein folds into one of two possible amphipathic alpha-helical conformations [106]. Conformation can be: uninterrupted N-terminal alpha-helix or two broken anti-parallel alpha-helices with the break between amino acids 44 and 45 [107]. Conformation depends on the underlying membrane curvature: the broken helix is formed on lipid surfaces with a high degree of curvature, while the uninterrupted helix is formed on less curved lipid surfaces [106, 108]. The N-terminal hydrophobic residues reside in the outer leaflet of the membrane, while the C terminus remains unfolded in both conformations [109]. These characteristics indicate that alpha-synuclein possesses the ability to be a membrane curvature sensor [109]. In addition, recent experiments using synthetic liposomes have shown that alpha-synuclein can also generate membrane curvature. [21, 110]

While this static model is well accepted it does not take into account the flux of alpha-synuclein between membrane-bound and free in the cytoplasm. Some evidence exists to suggest that some cytoplasmic machinery participates in the dynamic relationship between alpha-synuclein and the membrane. When artificial synaptic vesicles are bound to alpha-synuclein and cross-linked these assemblies become stabilized, suggesting that other proteins help to facilitate this interaction between lipid bilayer and alpha-synuclein [111]. The Tandon group from Toronto has very recently shown that, “anti-Rab3a antibodies strongly inhibited alpha-synuclein binding to
synaptosomal membranes” [100]. The GTP form of Rab3a co-immunoprecipitated with alpha-synuclein indicating that Rab3a machinery plays a role in the dynamic relationship between n alpha-synuclein and the phospholipid membrane [100]. Yet, the implication of this relationship and neurodegeneration remains to be elucidated.

**Figure #6:** Structure of alpha-synuclein revealing its amphipathic nature. Image procured from NCBI Structure, screen capture of Cn3D viewer 4.3.

**Aggregation of Alpha-synuclein**

Different conformational states and aggregated morphologies are possible for alpha-synuclein due to its intrinsically unfolded nature. These morphologies include: small aggregates, oligomers/multimers, protofibrils, β–sheet fibrils and α–helical structure when bound to membranes. Oligomer formation precedes fibril formation and
these oligomers have a wide variety of morphologies which depend on the incubation conditions [36]. Of the family of synucleins the alpha-synuclein protein is the most aggregation prone compared to β-synuclein and γ-synuclein [30].

Sequence analysis for the aggregation of alpha-synuclein has been an area of intense interest. The hydrophobic core of the protein, 35 amino acid NAC region, which was originally isolated from the brains of AD patients [104] plays an integral role in the aggregation process. While this central hydrophobic region facilitates aggregation the C-terminus of the protein inhibits aggregation and in fact when the C-terminus is truncated the abbreviated protein is more aggregation prone than the full-length protein [36]. Post-translational modifications of alpha-synuclein are numerous and the phosphorylation of Ser129 has been shown to increase the formation of fibrils [112]. When alpha-synuclein is bound to a membrane it has been shown to have an increased propensity to aggregate [113].

Currently, there is much debate as to which aggregate species is the most toxic. It is clear that the self-association tendencies of alpha-synuclein play a fundamental role in the neuronal dysfunction that is seen in PD and other synucleinopathies [114]. Some have suggested that the smaller oligomeric species are pathogenic while others have evidence that suggests the larger fibrillar species are pathogenic. Studying the conformational state of alpha-synuclein is notoriously difficult due to its inherently
unstructured nature and its ability to adopt a variety of conformations depending on experimental conditions [114]. Oligomeric species of alpha-synuclein are initially formed and considered to be relatively soluble [36]. Once the fibrillar state has been reached the structures become insoluble [36]. Insoluble fibrils have been detected in the brains of patients with PD via electron microscopy (EM) [73]. These filaments fall into two separate categories: linear as well as annular/circular and have a range of sizes that span from 50-700 nanometers (nm) [73]. Even oligomeric forms can attain a spherical morphology in the presence of divalent cations and these spherical oligomers have been shown to disrupt lipid bilayers [115]. Lipid bilayer disruption allows for an increase in cell permeability. Alpha-synuclein in the protofibrillar state can also form pore-like assemblies and these can act as an ion channels which may help explain some of the underlying vulnerability for these neurons to neurodegeneration (Figure #3) [116].

**Cell to Cell transfer of Alpha-synuclein**

Ten years ago Heiko Braak developed a staging method to describe the brain pathology seen in PD [117]. This staging method details the spatial and temporal spread of alpha-synuclein pathology of PD. In particular, the Braak method describes the presence of LB pathology (Figure #5) and correlation with PD symptom severity (Figure #7). Stage 1 describes LB and LN presence in the olfactory bulb and the vagus nerve [117, 118]. Stage 2 the pathology spreads toward the brainstem with involvement of the medulla oblongata specifically [117, 118]. Patients in these early stages do not exhibit
the motor symptoms typically associated with PD but may have some of the non-motor symptoms (anosmia, constipation). It is unclear if patients who have pathology at these stages are bound to progress and develop PD later in life. LB and LN pathology are found in the substantia nigra and amygdale in stage 3 [117, 118]. It is not until this stage that motor symptoms are expected to develop. Stage 4 sees the presence of LB and LN in the forebrain and cerebral cortex. Finally in stages 5 and 6 the pathology spreads toward the prefrontal cortex where its presence can contribute to the dementia that can be frequently found within the PD and DLB populations [117, 118]. From this correlative staging Braak hypothesized that there is a pathologic agent responsible for this type of gradual, infectious-like spread. Alpha-synuclein was quickly assigned the role of pathologic agent. Several cellular processes must be possible if alpha-synuclein can act as this prion-like pathologic agent. These cellular processes include: cellular release, cellular uptake, and most importantly the ability to “seed” pathology in a new cell.

In PD patients alpha-synuclein has been detected in the CSF as well as the plasma [51] suggesting that in the disease state affected cells may release alpha-synuclein even though it does not contain a secretory peptide sequence. Extracellular alpha-synuclein has also been found in the culture medium of human neuroblastoma cells as well as rat primary cortical neurons [119, 120]. Alpha-synuclein is secreted by non-classical exocytosis as it does not have a secretory peptide sequence. Following secretion from an initially affected cell alpha-synuclein must make its way to another cell for uptake. Neuronal cells in culture have demonstrated an endocytosis-dependent ability to take up
alpha-synuclein in both the monomeric form as well as the oligomeric and fibrillar forms [121]. Once transferred to another cell, alpha-synuclein needs to “seed” the endogenous intracellular alpha-synuclein. Recent experiments have shown this seeding pathology in a striking way. Desplats et al grafted murine neuronal progenitor cells into the hippocampus of mice that were expressing human alpha-synuclein and when the grafts were examined at one week post-transplant there was human alpha-synuclein staining present [122]. At 4 weeks, some of the graft contained alpha-synuclein aggregates which contained human alpha-synuclein. The Kordower group has shown that transplanted human fetal mesencephalic tissue into the SNpc of PD patients has resulted in accelerated pathological change in the neuronal cells found in the graft [48, 49]. Recently the Virginia Lee lab used mouse alpha-synuclein aggregates injected into the dorsal striatum and observed LB-like structures present in the striatum as well as other areas interconnected with the striatum [123]. There was a loss of dopamine and concomitant motor symptoms in these mice as well [123]. A caveat to this transfer quality has been uncovered; the recipient cells must express alpha-synuclein. When alpha-synuclein aggregates were injected into alpha-synuclein knockout mice there was a noticeable lack of aggregate propagation, confirming that endogenous alpha-synuclein expression is absolutely required for recruitment in this pathogenic process [124]. While the Braak hypothesis is still controversial, there seems to be a boon of evidence that describe the capacity of alpha-synuclein to act in a prion-like fashion.
**Figure #7**: Schematic of spatial and temporal spread of LB per the Braak hypothesis.

Adopted from [125]

Alpha-synuclein exhibits significant similarities to adenovirus protein VI

The literature exploring the molecular biology and pathological potential of alpha-synuclein is remarkably similar to studies exploring the mechanisms and pathological consequences of infection by non-enveloped viruses, specifically adenovirus. Because it lacks a membrane, adenovirus cannot rely on the fusion of viral and target cell
membranes to deliver its capsid and genome into the target cell cytoplasm. Instead, adenovirus utilizes a viral protein named protein VI (pVI) to achieve this goal. Following the endocytosis of adenovirus, pVI is released from the viral core and mediates the mechanical disruption of the endosomal/lysosomal (E/L) membrane [22]. Molecular studies of pVI have demonstrated that this protein is an amphipathic alpha-helix that possesses the ability to induce membrane curvature of endosomes and other experimental liposomes [126]. Notably, alpha-synuclein is also known to form an amphipathic alpha-helix in the presence of membranes (Figure #4) [18, 31, 106] and can induce membrane curvature [41, 42]. In fact, both pVI and alpha-synuclein induce a nearly identical disruption of artificial liposomes (Figure #8). Critically, the Wiethoff lab has demonstrated that disruption of endosome/lysosome (E/L) membranes by pVI results in an increase in mitochondrial ROS in target cells [27]. Cathepsin release from ruptured E/Ls mediates this effect, as ROS generation is inhibited by the cathepsin B inhibitor CA-074 [27, 127, 128]. The idea that alpha-synuclein may similarly rupture E/Ls, and thereby induce similar consequences, is the foundation of this proposal.
Figure #8: Disruption of experimental liposomes using aggregated alpha-synuclein in A as demonstrated by Varkey et al [42]. and adenovirus protein VI in B as demonstrated by Maier et al [126].

Significance

PD is the second most common neurodegenerative disease with an anticipated spike in patients due to the increasing longevity of the overall global population from the recent medical advancements made in other fields [4-7, 74]. Recent advances have made it clear that the protein alpha-synuclein plays a key role in the pathogenesis of this disease [3, 8, 9]. The structure of alpha-synuclein, an amphipathic alpha-helix, is an integral part of its ability to bind membranes and the similarity to adenovirus protein VI
is striking. The ability of alpha-synuclein to be secreted, taken up and seed pathology in cultured neurons as well as animal models lends authenticity to the prion-like hypothesis of alpha-synuclein propagation. Yet the exact mechanisms by which alpha-synuclein escapes from the endosome/lysosome remain largely unexplored. Thus the process of how an endocytosed protein becomes an intracellular protein has been the focus of this research. Given the importance of alpha-synuclein and how its molecular escape mechanism remains largely unexplored warrants deeper investigation.
CHAPTER THREE

ALPHA-SYNUCLEIN AGGREGATES CAN INDUCE LYSOSOMAL MEMBRANE RUPTURE AND RELEASE ACTIVE CATHEPSIN B

Abstract

PD as well as a host of other synucleinopathies have implicated a clear pathological role for alpha-synuclein. Alpha-synuclein has been shown to introduce an intracellular pathology that can be spread from cell-to-cell. However, the molecular mechanism by which alpha-synuclein induces this pathology is not well understood. Just as adenovirus ruptures intracellular vesicles we believe that alpha-synuclein operates similarly. In order to pursue this line of inquiry we had to categorize the effects on cells using various aggregate preparations. Aggregates were created using in-vitro purified recombinant protein subjected to incubations of varying length. Using a novel microscopy assay we were able to determine that intracellular vesicular rupture was occurring. Given the power of this assay we were able to determine which type of intracellular vesicle was being ruptured and the temporal relationship between the addition of alpha-synuclein aggregates and vesicular rupture. Due to the indication that the intracellular vesicle being ruptured was in fact a lysosome, and because lysosomes are replete with catalytic enzymes we endeavored to determine if the cathepsins being spilled into the cytosol were active. Given the important role in disease propagation,
progression, dysfunction and neurodegeneration, understanding the cellular mechanism of alpha-synuclein aggregates is of significant importance. In summary, our study demonstrates that aggregates induce the most rupture from the neuronal cells as soon as 24 hours after aggregate addition, and it is these aggregates that induce the rupture of lysosomes and the subsequent release of active cathepsin B.

**Introduction**

Numerous bodies of literature indicate that alpha-synuclein is clearly involved in the etiology of PD [8, 35, 55, 87, 93, 129-131]. Specifically, that neuronal pathology and cellular stress are increased in PD [8, 132-136]. *In vivo* and *in vitro* studies further demonstrate that acute exposure to alpha-synuclein aggregates lead to increased mitochondrial stress, endoplasmic reticulum (ER) stress, and problems associated with intracellular protein degradation machinery. Closely related in structure and membrane affinity to adenovirus protein VI, alpha-synuclein is a protein that clearly has the possibility to rupture intracellular vesicular membranes. Despite the overwhelming amount of evidence indicating the structural similarity of these two proteins, only a small number of studies have examined the effects of alpha-synuclein on membranes, specifically intracellular vesicle membranes [16, 41, 42, 100, 106, 107, 109, 115].

In addition to the toxic oligomeric form of alpha-synuclein that is retained in neurons, a significant amount of alpha-synuclein is released from cells and can be taken
up via endocytosis into recipient cells [122, 137]. Despite knowing how cells take up alpha-synuclein aggregates, not much is known about these aggregates once they have been internalized. Due to their ability to bind and bend membranes, [16, 41, 42, 100, 105-107, 109] as well as their presence in the cytosol, it has been hypothesized that intracellular vesicle rupture occurs. Fortunately, an assay in the field of virology has been developed for the specific purpose of monitoring intracellular vesicle membrane integrity [22, 27, 126-128]. This assay was developed exploiting the biological properties of a protein called galectin-3 (gal-3). Very recently, Paz et al was able to identify gal-3 as a cytosolic protein that could act as a marker for ruptured phagosome membranes [138]. Gal-3 is a member of the galectin family of proteins, which all members contain a consensus sequence in its carbohydrate recognition domain (CRD) [139-141]. This consensus sequence has a distinct affinity for β–galactoside containing glycoconjugates [139-141]. N-linked glycans, a type of β–galactoside containing glycoconjugates, are found on the exterior leaflet of the plasma membrane as well as the interior leaflet of any vacuole/vesicle that is involved in the endocytic process. Immediately following membrane disruption the cytosolic gal-3 is recruited to the area where the N-linked glycans are exposed from the interior vacuole surface following lysis [138]. Immunofluorescence microscopy (IFA) can be used to monitor membrane rupture when gal-3 is fluorescently tagged. In the absence of rupture gal-3 staining appears diffuse in the cytosol, whereas the fluorescent signal appears punctuate after being recruited to a ruptured membrane (Figure #9) [138]. Maier et al recently used this
technique to demonstrate that adenovirus type 5 (Ad5) is able to mechanically disrupt endosomal membranes to gain access to the cytosol [126]. Galectin-8 and galectin-9 have also been shown to be recruited to ruptured membranes [54, 142].

Figure #9: Schematic of galectin-3 localization with intact vesicular membranes and with ruptured vesicular membranes. [143]

Currently, the literature addressing intracellular membrane rupture of alpha-synuclein is sparse. It has been shown that uptake of alpha-synuclein aggregates is attenuated by low temperature and inhibitors of dynamin [120, 144]. Alpha-synuclein
has also been shown to colocalize with Rab5a and Rab7, with dominant negative Rab5a models showing reduced endocytosis of alpha-synuclein [122]. Also, when cells are treated with trypsin, removing endocytic receptor proteins, the uptake of alpha-synuclein is inhibited [145]. In fact a recent review by the Brundin group asks “how does alpha-synuclein leave the endosome to access the cytosol?” [146]. Meanwhile, the Thurston group has shown that gal-3, gal-8 and gal-9 are recruited to LAMP1 positive vesicles, indicating that Salmonella Typhimurium ruptures the lysosomal membrane [54]. Gal-3 can be used as a sensor for E/L rupture and further investigation of which intracellular vesicle is ruptured will be helpful. Rupture of the lysosome will result in the possible activation of multiple downstream pathways. Cahepsins are tightly regulated cellular proteins which are generated as zymogens that are activated once a low pH has been reached in the endosomal/lysosomal compartment. The release of cathepsin from these compartments post-activation would lead to unregulated intracellular damage due to the cleavage of cellular proteins creating an impairment of cellular functions [147]. Depending on the degree of cathepsin dysregulation, effects can range from generation of ROS to the induction of apoptosis, both of which are well established aspects of PD pathology [27, 148].

To explore the impact of aggregated alpha-synuclein on intracellular vesicle membrane integrity, our study utilized two neuronal cell lines: N27, a rat dopaminergic cell line; and SH-SY5Y, a human neuroblastoma cell line. Both cell lines have been
stably transduced to express mcherry labeled galectin-3 (chgal-3) in order to evaluate the spatial and temporal characteristics of alpha-synuclein aggregates on intracellular vesicle membrane integrity. Using differentiated neuronal cell lines, our study models a scenario in which the neurons are challenged with a variety of different sized aggregated alpha-synuclein. We also sought to determine if there is a temporal relationship between vesicle rupture and aggregate addition as well as any downstream consequences of that rupture. Herein, we are able to show that exposure to alpha-synuclein aggregates results in a membrane perturbation of both cell lines at twenty-four hours of exposure to aggregates. Moreover, our data suggests that alpha-synuclein aggregates have more profound effects on late-stage lysosomes than endosomes. Lastly, we demonstrate that cathepsin B is released from these late-stage lysosomes.

Materials and Methods

Cell Lines

The human neuroblastoma cell line, SH-SY5Y was obtained from the American Type Culture Collection (ATCC). The rat dopaminergic neuronal cell line was a kind gift from Dr. Anumantha Kanthasamy. SH-SY5Y cells were maintained and differentiated with 50 mM all L–trans Retinoic acid every 48–72 hours in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS)(Hyclone) and 100 IU/mL penicillin, 100 µg/mL streptomycin and 10 µg/mL ciprofloxacin. The rat
dopaminergic cell line N27 was cultured in RPMI media supplemented with 10% fetal bovine serum (FBS)(HyClone) and 100 IU/mL penicillin, 100 µg/mL streptomycin and 10 µg/mL ciprofloxacin. Cells were maintained in a 37°C incubator with 5% CO2. The mCherry-gal3 plasmid was a generous gift from Dr. Christopher Wiethoff. Retroviral vectors were generated utilizing transfection of 293T cells with the packaging plasmids vesicular stomatitis virus glycoprotein (VSV-g), pCigB and mCherrygal-3. Transfections were performed using polyethylenimine (PEI) (molecular weight, 25,000; Polysciences) as previously described [149]. Supernatants containing these recombinant retroviral vectors were collected 48 hours post-transfection and used to transduce SH-SY5Y and N27 cells. Positive transductants were selected with Geneticin/G418.

Alpha-synuclein

Full length alpha-synuclein and the E46K missense mutant alpha-synuclein were purchased (rPeptide) and the lyophilized protein was rehydrated in PBS with 100 mM NaCl immediately upon arrival to a concentration of 1 mg/ml and was aliquoted and stored at -80°C. In order to generate aggregates, alpha-synuclein was incubated for 3 days at 37°C under constant agitation at 300 revolutions per minute (rpm) followed by storage at -80°C. Aggregates were added at a concentration of 3µg/ml per the protocol provided by Volpicelli-Daley [150]. Aggregates were fluorescently labeled with Dylight® 488 N-hydroxysuccinimide (NHS)-ester fluorophores (Thermo Scientific), according to the manufacturer's protocol prior to use. Briefly, the aggregates were dialyzed into a sodium-
phosphate buffer with pH 8.0. After dialysis the aggregates were labeled with the Dylight® 488 for thirty minutes. The reaction was quenched using 40mM Tris and dialyzed again. These aggregates were then stored at \(-20^\circ\)C.

**Coomassie**

Samples were run on a native 10% polyacrylamide gel. Gel was fixed using colloidal coomassie fixative (45% methanol and 10% acetic acid) for 1 hour. Gel was then placed in Coomassie Brilliant Blue G-250 (MP Pharmaceuticals) overnight at room temperature. The following morning the gel was imaged using a BioRad ChemiDoc XRS imaging system (BioRad).

**Western Blot Analysis**

Purified proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis, (SDS-PAGE), proteins were transferred to nitrocellulose membranes and detected by incubation with the primary antibody. The H3C monoclonal antibody developed by Julia George was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Secondary antibody conjugated to HRP (Thermo Scientific) was used where necessary, and antibody complexes were detected using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific).
Chemiluminescence was measured using a BioRad ChemiDoc XRS imaging system (BioRad).

*Glutaraldehyde Cross-linking*

Equivalent amounts of aggregated alpha-synuclein and monomeric alpha-synuclein were incubated with 0, 1, and 2 μM glutaraldehyde for 15 minutes at room temperature. 1 M glycine was added after 15 minutes to saturate the glutaraldehyde. 2× SDS sample buffer was added and the mixture was boiled for 5 min at 100°C. The samples were then subjected to SDS-PAGE using 4%–15% Tris–HCl gradient gels (Ready Gels, BioRad) and subsequent Western Blot analysis.

*K114*

The K114 fluorescent amyloid fibril binding assay was performed in 100 mM glycine buffer, pH 8.5 with 100 μM K114 (Tocris Bioscience) using an Olis-DM 45 spectrofluorometer. Fluorescence was measured with an excitation wavelength of 380 nm, and emission wavelength of 550 nm with a cutoff established at 530 nm for all assay endpoints.

*Electron Microscopy*
Alpha-synuclein was added to carbon coated, 200mesh grids for 90 seconds. Grids were stained with 2% uranyl acetate for 45 seconds. Grids were dried overnight and imaged on a Hitachi H-600 transmission electron microscope (TEM).

**Immunofluorescence Microscopy**

Cells were allowed to adhere to Fibronectin (Sigma-Aldrich) treated glass coverslips and fixed with 3.7% formaldehyde (Polysciences) in 0.1 M piperazine-N,N'bis(2-ethanesulfonic acid) PIPES buffer at pH 6.8 for 15 min. Cells were then permeabilized for 20 minutes in PBS with 10% Normal Donkey Serum (NDS) and 0.5% saponin (Sigma-Aldrich). Staining with specific monoclonal or polyclonal antibodies was done in 10% NDS with 0.5% saponin for 20 minutes. Mouse-anti alpha-synuclein (BD Biosciences), EEA-1 (BD Biosciences), and LAMP-2 (BD Biosciences) were immunostained at 1:400 dilutions. Primary antibodies were secondarily labeled with fluorophore-conjugated donkey anti-mouse or donkey anti-rabbit antibodies (Jackson ImmunoResearch). Images were collected with a DeltaVision microscope (Applied Precision) equipped with a digital camera (CoolSNAP HQ; photometrics), using a 1.4-numerical aperture (NA) 100X objective lens, and were deconvolved with SoftWoRx deconvolution software (Applied Precision). Structured Illumination images were collected on an OMX microscope (Applied Precision) and deconvolved and reconstructed using SoftWoRx software (Applied Precision). Tiff images and 3-dimensional reconstructions were generated using Imaris software (Bitplane).
**Image Analysis**

Deconvolved images were analyzed for colocalization of Galectin-3 (Gal-3) with LAMP2 and Early Endosome Antigen-1 (EEA-1) signal by use of the Surpass Mode of the Imaris software package (Bitplane).

**Statistical Analysis**

Results are presented as mean ± standard error of the mean. ANOVA was used to compare between different experimental groups. All statistical analyses were performed using InStat 3.0 (GraphPad Software). \( p<0.05 \) was considered statistically significant.

**Results**

*Characterization of Alpha-synuclein Aggregate Preparations*

Alpha-synuclein aggregates were generated by resuspending recombinant lyophilized protein followed by agitation for three days at 37°C. Prior to agitation, purified recombinant alpha-synuclein ran at a molecular weight of slightly less than 50 kD on a non-denaturing gel. The three day incubation altered the electrophoretic mobility of the protein in a non-denaturing gel in two ways. First, the primary band in the solution exhibited less electrophoretic mobility than the presumably monomeric form of
the protein (Figure 1A). Second, we also observed the appearance of a number of bands which migrated below the primary band. Both of these changes may represent altered structural changes in alpha-synuclein induced during incubation or may also represent a multimeric form of alpha-synuclein which is induced during incubation. Under denaturing conditions, all of these bands resolved to the expected ~15 kilodalton (kD) molecular weight of alpha-synuclein (Figure #10A). We also performed glutaraldehyde crosslinking to further define the high molecular weight species of alpha-synuclein induced by our incubation conditions. Incubation induced the formation of high molecular weight species of alpha-synuclein observable following glutaraldehyde crosslinking (Figure #10B). These higher molecular weight species were not observed following glutaraldehyde crosslinking of freshly resuspended alpha-synuclein (Figure #10B). We also characterized our incubated protein with the dye K114, which can be used to measure amyloid fibrils in solution. We observe that incubation induced the formation of fibrillar structures, as measured by an increase in K114 fluorescence relative to freshly resuspended alpha-synuclein (Figure #10C) (p<0.01). Similarly, transmission electron microscopy of wt alpha-synuclein (with or without a 3 day incubation at 37°C) revealed fibrillar structures only following incubation. No such structures were detected in the absence of incubation (Figure #10D). These analyses revealed that this protocol induces a heterogeneous combination of fibrillar and oligomeric species of alpha-synuclein. We will refer to this heterogeneous population of alpha-synuclein species as “aggregates”.
Figure #10: Alpha-synuclein aggregate characterization. A. Wild-type alpha-synuclein aggregates were generated using in vitro purified protein. Recombinant lyophilized alpha-synuclein was resuspended and followed by constant agitation for three days at 37°C. The aggregates generated in this fashion were run on a non-denaturing gel (left) or denaturing gel (right), fixed and stained with Coomassie brilliant blue. B. Following incubation for 3 days as described, alpha-synuclein preparations were fixed with glutaraldehyde at the indicated concentration for 15 minutes at room temperature. C.
K114 analysis of alpha-synuclein which was freshly resuspended or incubated as described. Emission was measured at 550 nm. Error bars represent the standard error of the mean of 3 readings. Results are representative of at least three independent experiments (* indicates a P value < .01). D. Transmission electron micrograph of alpha-synuclein preparations from freshly resuspended protein or after 3 days of incubation as described. Scale bars = 500 nm.

*Alpha-synuclein aggregate exposure leads to rupture of endocytic vesicles*

Galectin-3 is a sugar binding protein which recognizes beta-galactoside sugars which are normally only present on the exterior leaflet of the plasma membrane and the interior leaflet of intracellular vesicles [151]. Galectin-3 relocalization has therefore been utilized as a tool to identify ruptured vesicles in studies of bacteria and viruses which rely on vesicle rupture to enter the cytoplasm during infection [54, 126, 142]. To test the hypothesis that alpha-synuclein can induce the rupture of intracellular vesicles following endocytosis, we transduced human SH-SY5Y neuroblastoma cells and the rat dopaminergic neuronal N27 cell line with a retroviral vector expressing mCherry-Galectin3 (chGal3). We treated these cells with the alpha-synuclein aggregates described in Figure #10. Incubation of N27 or SH-SY5Y cells stably expressing mCherry-Gal3 (N27chGal3, SY5YchGal3) with alpha-synuclein aggregates induced a pronounced redistribution of chGal3 in both cell lines. While in the absence of aggregate exposure, chGal3 maintained a diffuse cytoplasmic localization in both cell lines; incubation with
alpha-synuclein aggregates for 24 hours induced the relocalization of chGal3 to intracellular, punctate structures (Figure #11). This relocalization suggests that alpha-synuclein aggregates are able to disrupt the integrity of intracellular vesicular membranes following endocytosis. Treatment of cells with freshly resuspended (monomeric) alpha-synuclein did not induce relocalization of chGal3 (Figure #12), suggesting that non-monomeric forms or aggregated forms of alpha-synuclein are responsible for vesicular rupture under these conditions.
**Figure #11:** Alpha-synuclein aggregates induce discrete Galectin-3 puncta: SH-SY5Y and N27 cells stably expressing chGal3 were treated with alpha-synuclein aggregates for 24 hours. In both cell lines a relocalization from the diffuse, untreated state can be clearly visualized after 24 hours. This relocalization is indicative of intracellular vesicular rupture. Images are representative of at least three independent experiments.
Figure #12: Alpha-synuclein monomers do not induce Galectin-3 localization:

N27 and SH-SY5Y cells stably expressing chGal3 were incubated with freshly resuspended alpha-synuclein for 24 hours. Treatment of these cells with freshly resuspended alpha-synuclein did not induce the redistribution observed at an equivalent concentration of alpha-synuclein aggregates (see Figure #11).
Alpha-synuclein aggregates induce the rupture of lysosomes

Previous studies of adenovirus found that protein VI mediated vesicle rupture occurs more frequently in vesicles positive for the endosomal marker Early Endosome Antigen 1 (EEA1), than in those positive for the lysosomal marker, Lysosomal Associated Membrane Protein-1 (LAMP-1) [126]. We similarly assessed the nature of the ruptured vesicles induced following alpha-synuclein incubation. Unlike the case of vesicles ruptured by protein VI, we observed the vast majority of chGal3 positive vesicles induced following incubation with alpha-synuclein aggregates were positive for the lysosomal marker, LAMP2 (Figure #13A), while very few chGal3 positive vesicles showed EEA1 staining above background (Figure #13B). This observation was confirmed by quantitative analysis of the amount of LAMP2 or EEA1 signal present in individual chGal3 puncta (7.43% of chGal3-positive vesicles exhibited EEA1 staining above background levels, whereas 91.98% of these vesicles exhibited LAMP2 staining greater than secondary antibody alone (Figure #13C). This suggests that, following endocytosis, alpha-synuclein induces the rupture of vesicles which are predominantly lysosomes, in contrast to the case of protein VI, which more frequently ruptures vesicles containing positive for EEA1 [126].
**Figure #13:** *Alpha-synuclein aggregates induce lysosomal rupture.* A. SH-SY5Y chGal3 cells (red) treated with alpha-synuclein aggregates exhibit colocalization between chGal3 and LAMP2 (green). The area shown in the white box in the upper left panel is enlarged in the other three panels. B. SH-SY5Y chGal3 cells (red) treated with alpha-synuclein, which does not induce colocalization between chGal3 and EEA1 (green). The area shown in the white box in the upper left panel is enlarged in the other three panels. C. Quantitative analysis of the colocalization of fluorescent intensity of EEA1 or LAMP2 staining in individual chGal3 puncta identified using Imaris image analysis software. 7.43% of chGal3-positive vesicles exhibited EEA1 staining above background levels, whereas 91.98% of these vesicles exhibited LAMP2 staining greater than secondary antibody alone. Forty images were collected for each group. More than 190 individual puncta were analyzed for each group.

*Alpha-synuclein localizes to areas of vesicular rupture in cells*

We next determined if alpha-synuclein localized to ruptured vesicles by directly labeling alpha-synuclein aggregates with the Dylight 488 N-hydroxysuccinimide (NHS)-ester [137]. When fluorescently labeled alpha-synuclein aggregates were added to SY5Y chGal3 cells, ruptured vesicles containing alpha-synuclein were clearly evident in these cells 24 hours later (White Arrows, **Figure #14**). However, we also observed chGal3 puncta which did not contain detectable alpha-synuclein signal (Red Arrow, **Figure #14**). This association of alpha-synuclein with ruptured vesicles was confirmed in live cell
imaging experiments, in which alpha-synuclein and chGal3 remain associated during brief intracellular trafficking events (Movie S1).

**Figure #14:** Labeled alpha-synuclein colocalizes with chGal-3 in neuronal cell lines. SH-SY5Y chGal3 cell exposed to Dylight 488 conjugated alpha-synuclein aggregates for 24 hours. The white box in the upper left panel is enlarged in the other three panels to demonstrate colocalization of alpha-synuclein and chGal3. White arrows show vesicles demonstrating colocalization between chGal3 and alpha-synuclein. The red arrow highlights a ruptured vesicle that does not appear to contain alpha-synuclein.
To better assess the localization of alpha-synuclein within ruptured vesicles, we performed structured illumination microscopy (SIM). SIM utilizes pattern based imaging of a specimen to generate reconstructions of the specimen with resolutions below the diffraction limit of light microscopy (~200 nm) [22]. When reconstructions of N27 cells incubated with alpha-synuclein were analyzed, alpha-synuclein was frequently observed to associate with the periphery of chGal3 positive vesicles, frequently forming arced or circular localizations (Figure #15). In some cases, alpha-synuclein formed an individual arc at the periphery of discrete ruptured vesicles (panel 2, Figure #15). In other cases, alpha-synuclein clustered in a series of circular localizations suggesting the presence of alpha-synuclein in a multivesicular compartment (panel 1, Figure #15). Additional examples of the intravesicular localization of alpha-synuclein are shown in Figure S2.

The arced localization of alpha-synuclein at the vesicular periphery was also evident in three dimensional modeling of these reconstructions (Movie S2). This is consistent with the idea that luminal alpha-synuclein induces membrane curvature that causes the rupture of these vesicles. Collectively these results demonstrate that following entry into target cells, alpha-synuclein aggregates are present in endocytic vesicles which have been ruptured. As these vesicles are not present in the absence of alpha-synuclein aggregates, we conclude that alpha-synuclein mediates the rupture of these vesicles.
Figure #15: Structured Illumination Microscopy (SIM) of alpha-synuclein mediated vesicle rupture. N27chGal3 cells were exposed to Dylight 488 conjugated alpha-synuclein aggregates for 48 hours. Cells were fixed and imaged using a structured illumination fluorescent microscope, allowing the images to be reconstructed with a resolution below the diffraction limit of light microscopy. The boxed area in the left panel is enlarged in the two panels to reveal the intraluminal localization of alpha-synuclein.
CHAPTER FOUR

FOLLOWING LYSOSOMAL RUPTURE, AGGREGATED ALPHA-SYNUCLEIN INDUCES A CATHEPSIN B DEPENDENT INCREASE IN REACTIVE OXYGEN SPECIES (ROS)

Abstract

Here we examine the downstream consequences of the addition of alpha-synuclein aggregates. A critical aspect of our model of the cellular mechanism for PD pathogenesis is the connection between E/L rupture and ROS induction. Lysosomal rupture may be an early event in the apoptotic cascade and mitochondrial release of pro-apoptotic factors may be a consequence of previous lysosomal destabilization [152]. It has been shown that the rupture, particularly of lysosomes, leads to an induction of ROS in a cathepsin dependent manner [27, 152]. We are able to show in both neuronal cell lines an increase in the generation of ROS along a temporal scale similar to that observed in gal-3 puncta formation. Aggregate size also played a role in determining ROS generation. This increase in ROS is shown to be almost completely cathepsin B dependent as pre-administration of a selective cathepsin B inhibitor ameliorates the several fold increase seen in ROS. Demonstration that the ROS being generated was specific to mitochondria was shown by using a redox-sensitive form of Green Fluorescent Protein (GFP) (RO-GFP).
**Introduction**

Intracellular rupture of lysosomes leads to the release of cathepsins in addition to other proteins as well. Lysosomes are organelles that contain a wide variety of proteases (cathepsins) that help to maintain the turnover of membrane proteins and other macromolecules found in the cell [153]. Lysosomal processing capacity diminishes progressively over the lifespan of an animal [154, 155], and lysosomal malfunction is associated with age-related neurodegenerative disorders [155-158]. Inhibition of lysosomal enzymes has been shown to lead to abnormal protein accumulation and aggregation, synaptic loss, as well as neurodegeneration in laboratory models [159-161].

Addition of purified cathepsin B and cathepsin D can generate significant increases in mitochondrial ROS production [152]. Mitochondrial dysfunction in PD has also had the attention of the PD field in recent years [71, 162-164]. In addition to MPP⁺ there are several other environmental toxins that have the ability of to target mitochondria to create Parkinsonian symptoms [165, 166]. In human PD patient tissue samples of the SNpc there is a substantial decrease in the activity of mitochondrial complex I of the electron transport chain (ETC) [167]. This decrease in activity is thought to be specific to PD patients and seems to reflect oxidative damage to complex I [168, 169]. Other cellular components in the SNpc of PD patients: lipids, proteins and DNA also have been found to be damaged by oxidative stress [170]. The source of this oxidative stress is largely mitochondrial. For the majority of eukaryotic cells, the electron transport chain
(ETC) is found in the mitochondrial membrane. Even under basal conditions, the ETC can leak up to 2% of all electrons as ROS, and when cells are exposed to a number of extracellular stressors intracellular production of ROS increases [152].

The physical proximity of mitochondrial DNA (mtDNA) to the site of ROS generation probably makes them an even more vulnerable target. The mitochondrial genome encodes 13 proteins involved in the mitochondria respiratory chain, 7 of which are involved in the formation of complex I. The number of mtDNA mutations present in clonally expanded clusters of SNc mitochondria is positively correlated with age and negatively correlated with cytochrome oxidase activity (a marker for functional respiratory activity). Their clonal nature argues that these mutations are due to the expansion of a somatic mutation, not a genetic mutation present at birth. Because many of these mtDNA mutations impair ETC function, they are likely to contribute to the loss of SNc DA neurons. [71]

There seems to a very plausible connection between lysosomal rupture and mitochondrial oxidative stress.

Cathepsins have been shown to have a decreased immunofluorescent staining in post-mortem PD brains when compared with age-matched controls [153]. This alteration in cathepsin level was found to be most prominent in neurons found in the SNpc that contained alpha-synuclein inclusionis [153]. This led us to hypothesize that one of the
cathepsins may be playing a role in the downstream consequences of alpha-synuclein aggregate inducing lysosomal rupture. In order to measure the ROS level of the two neuronal cell lines we used 2', 7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA), a cell-permeant dye. It is a fluorescein derivative that has its acetate groups cleaved by intracellular esterases and when oxidized it becomes converted to a highly fluorescent version. The presence of fluorescence is indicative of ROS detection. While this is a reliable test, it does not specifically measure ROS generated by the mitochondria. In order to further validate our findings as well as obtain more specific data concerning the location of ROS generation we used reduction-oxidation sensitive green fluorescent protein (roGFP) [171, 172]. This protein uses a modified version of GFP. It has a mitochondrial targeting sequence with two cysteine residues in the beta-barrel structure. The oxidation state of each cell can be individually measured using roGFP as the oxidation state of the thiols present in the roGFP molecule will determine the fluorescent properties of the sensor.

Material and Methods

Cell Lines and reagents

The human neuroblastoma cell line, SH-SY5Y, and the human monocyte cell line THP-1 were obtained from the American Type Culture Collection (ATCC). The rat dopaminergic neuronal cell line was a kind gift from Dr. Anumantha Kanthasamy [16].
SH-SY5Y cells were maintained and differentiated with 50 mM all L–trans Retinoic acid every 48–72 hours in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 100 IU/mL penicillin, 100 µg/mL streptomycin and 10 µg/mL ciprofloxacin. THP-1 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media, supplemented with 10% FBS, 100 IU/ml penicillin, 1 mg/ml streptomycin, 0.25 µg/ml amphotericin B, non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES buffer and 2 mM glutamine. The rat dopaminergic cell line N27 was cultured in RPMI media with the same additives used in the DMEM. Cells were maintained in a 37°C incubator with 5% CO2.

**Alpha-synuclein**

Full length alpha-synuclein and E46K mutant alpha-synuclein were purchased (rPeptide) and the lyophilized protein was rehydrated to a concentration of 1 mg/ml. alpha-synuclein was incubated for 3 days at 37°C in PBS with 100 mM NaCl under constant agitation followed by aliquoting and storage at −80°C. Aggregates were fluorescently labeled with Dylight 488 N-hydroxysuccinimide (NHS)-ester fluorophores (Thermo Scientific), according to the manufacturer’s protocol prior to use.

**Live Cell Imaging**
Images were acquired on a Deltavision deconvolution microscope equipped with a Weather Station™ chamber utilized to maintain the cells at 37°C at 5% CO2. Images were acquired using a Cascade 2 EMCCD (Photometrics) and deconvolved and reconstructed using SoftWoRx software (Applied Precision). Tiff images and 3-dimensional reconstructions were generated using Imaris software (Bitplane).

**ROS Assay**

All N27 and SH-SY5Y cells were plated at 100,000–200,000 cells per well in a black 96-well plate (Costar) in RPMI or DMEM with 10% fetal bovine serum (FBS). Following 1 hour of serum starvation the cells were incubated with the ROS-sensitive fluorophore 2, 7-dichlorofluorescein diacetate H₂DCFDA (DCF; Invitrogen) at 10 µM for 1 hour per the manufacturer's protocol followed by two washes with phosphate-buffered saline (PBS). If pretreated with cathepsin B inhibitor, cells were incubated with 100 µM CA-074Me (EMD Biosciences) or dimethylformamide (DMF) as vehicle control for 1 hour following H₂DCFDA incubation. Alpha-synuclein aggregates were added at a concentration of 3 µg/ml in accordance with previous concentrations and the Volpicelli-Daley protocol [150]. Fluorescence intensity of oxidized DCF was measured over the course of 72 hours, at an excitation wavelength of 485 nm and an emission wavelength of 520 nm, on a fluorescent plate reader (Biotek). Results are presented as background subtracted values (background was defined as cells that were not loaded with DCF).
RoGFP assessment of mitochondrial ROS

RoGFP2 fused to a mitochondrial targeting sequence (mRoGFP) was obtained from S James Remington (University of Oregon). The open reading frame was inserted into lentiviral vectors containing a puromycin resistance cassette and vector stock was used to transduce parental SH-SY5Y cells. Cells stably expressing mRoGFP were selected in DMEM containing 5 µg/ml puromycin. Cells were imaged in DMEM lacking phenol red (Invitrogen) at 90 second intervals. The basal oxidation state of individual cells was assessed for 6 minutes. 4 mM Dithiothreitol was added after 6 minutes to define the minimum oxidation state of the imaged cells. After 6 minutes (4 time points) in DTT, the cells were quickly washed with PBS and 300 µM Aldrithiol was added to define the maximal oxidation state of the imaged cells. The relative oxidation state of each cell was calculated by fixing the minimum oxidation state observed in the presence of DTT to 0 and the maximum oxidation state observed in the presence of Ald to 1, as previously described by Guzman et al [173]. Averages are from an analysis of a minimum of 15 cells from each treatment group that was analyzed.

Results

Alpha-synuclein aggregates induce a cathepsin dependent increase in ROS

The rupture of endosomes and lysosomes is associated with an increase in cellular ROS in cells [27, 174-176]. Therefore we asked if vesicle rupture by alpha-synuclein
aggregates could similarly induce an increase in ROS in cells. We utilized the redox sensitive fluorophore H2DCFDA to monitor the ROS generated in neuronal cells following treatment with alpha-synuclein aggregates. Treatment with alpha-synuclein aggregates induced a significant (P<0.05 1-way ANOVA with Bonferroni post-hoc) increase in the fluorescence observed in both the N27 and SH-SY5Y cells (Figure #16A, 16B). To determine if this increase in ROS was related to the release of activated cathepsins from ruptured lysosomes, we incubated cells with alpha-synuclein aggregates in the presence of the cathepsin B inhibitor, Ca074-me. Ca074-me significantly abrogated the increase (P<0.01) in ROS observed following treatment with alpha-synuclein aggregates (Figure #16A, 16B). These experiments collectively demonstrate that alpha-synuclein can induce a cathepsin B dependent increase in ROS in target cells. In order to corroborate this data, we utilized SH-SY5Y cells stably expressing a redox sensitive form of GFP [171, 172] targeted to mitochondria to determine if the ROS induced by alpha-synuclein specifically altered the redox state of the mitochondrial membrane in affected cells. This protein allows for the measurement of the mitochondrial oxidation state in living cells relative to the minimum oxidation, which is measured by the addition of a strong reductant, dithiothrietol (DTT), and to the maximum oxidation, which is measured by the addition of a strong oxidant, aldrithiol [173]. The initial oxidation state for each cell can then be calculated relative to the minimal and maximal oxidation states defined by these two conditions. As shown in Figure #16C, cells treated with alpha-synuclein aggregates exhibited increased mitochondrial oxidation relative to control treated cells.
**Figure #16:** Alpha-synuclein aggregates induce a Cathepsin-B dependent increase in ROS. SH-SY5Y (A) or N27 (B) cells loaded with the fluorescent substrate H2DCFDA reagent and incubated in the presence or absence of alpha-synuclein aggregates with or without treatment with the cathepsin B inhibitor Ca-074Me. Error bars represent the SEM of triplicate samples. C. SH-SY5Y cells stably expressing mitochondrially targeted roGFP1 were incubated for 72 hours with alpha-synuclein aggregates and the oxidation state of the mitochondria of individual cells was assessed at described in the text. Error bars represent the SEM of 15 or more cells analyzed for each treatment group. * p<0.001.

*Larger aggregates generated by the E46K alpha-synuclein mutant fail to induce vesicular rupture and ROS production*

We next examined the ability of the genetically-linked PD alpha-synuclein mutant, E46K, to induce vesicular rupture and ROS in target cells. When purified, recombinant E46K alpha-synuclein was subjected to the same incubation protocol as wild-type (wt) the E46K mutant exhibited reduced electrophoretic mobility compared to wt aggregates (**Figure #17A**). As with the wt, all of these bands resolved to the expected ~15kD band under denaturing conditions (data not shown). Glutaraldehyde crosslinking revealed that higher molecular weight species of E46K alpha-synuclein were also present following incubation (**Figure #17B**). K114 analysis revealed that the E46K mutant had
more fibrillar content that wt alpha-synuclein incubated under the same conditions (p<0.01) (**Figure #17C**). Consistent with this observation, macroscopically visible aggregates were present in the E46K samples following incubation (data not shown). Fibrillar species were also readily detectable in these preparations using electron microscopy (Figure S3D). Thus, in following our aggregation protocol, E46K alpha-synuclein formed fibrillar structures to a greater degree than that observed for wt alpha-synuclein.
Figure #17: *E46K aggregate characterization.* E46K mutant alpha-synuclein was generated using in-vitro purified protein. Recombinant lyophilized E46K alpha-synuclein was resuspended and constantly agitated for three days at 37°C. A. The aggregates generated in this fashion were run on a non-denaturing gel, fixed and stained with
Coomassie brilliant blue. E46K alpha-synuclein ran at a higher molecular weight than the wild-type alpha-synuclein on the non-denaturing gel. B. Following incubation for 3 days as described, E46K alpha-synuclein preparations were fixed with glutaraldehyde at the indicated concentration for 15 minutes at room temperature. C. The fibrillar content of E46K aggregates was assessed using K114 staining. E46K aggregates had significantly more fibrillar content than wt aggregates following identical treatement (*P<0.01). Results are representative of at least three independent experiments D. TEM image of E46K alpha-synuclein fibril

We next asked if E46K alpha-synuclein preparations were as competent at wt alpha-synuclein to induce ROS in target cells. E46K alpha-synuclein aggregates induced significantly less ROS than wt alpha-synuclein in N27 and SH-SY5Y cells (Figure #18A, 18B), (p = 0.039 in N27, p = 0.024 in SY5Y). However, this degree of ROS induction was still significant relative to control treated cells (p<0.001 in N27 p<0.016 in SY5Y). When cells treated with these E46K alpha-synuclein aggregates were analyzed by fluorescent microscopy, we observed that a significant amount of the alpha-synuclein stain localized to large aggregates that appeared too large to enter the cell via endocytosis. These aggregates did not induce chGal3 relocalization (Figure #18C).

Collectively, these results demonstrate that large alpha-synuclein amyloid aggregates do not induce chGal3 relocalization and preparations which contain larger protein aggregates are less effective at inducing ROS in target cells. The most likely explanation for this
observation is that large aggregates are unable to enter the endocytic compartment and therefore do not induce the rupture of lysosomes and subsequent cathepsin dependent mitochondrial stress.

**Figure #18:** Large aggregates generated by E46K alpha-synuclein mutant are unable to efficiently induce vesicle rupture and ROS. (A) SH-SY5Y or (B) N27 cells were loaded with H2DCFDA reagent and incubated with wt or E46K alpha-synuclein. Error bars
represent the SEM of triplicate samples. Results are representative of at least three independent experiments. C. Purified recombinant E46K alpha-synuclein aggregates were added to N27 chGal3 cells for 24 hours, stained with an alpha-synuclein specific antibody (green) and imaged as in previous experiments. Results are representative of at least three independent experiments.
CHAPTER FIVE

GALECTIN-3 MAY BE PLAYING A ROLE IN THE CELLULAR SECRETION MECHANISM OF ALPHA-SYNUCLEIN

Abstract

In order to perpetuate the PD pathology, alpha-synuclein must be spread in cell-to-cell manner. This would create a positive feedback loop of neurodegeneration. We have shown that aggregated alpha-synuclein induces lysosomal rupture and generates ROS as part of the downstream effects. For a molecule to be an agent of pathology it must exit the host cell and complete a transfer from the first cell to the second cell, gaining access to the second cell by crossing the plasma membrane. Alpha-synuclein has shown the ability to exit from cells utilizing various mechanisms, specifically exocytosis. Different aggregation state of alpha-synuclein have been discovered in CSF and in blood plasma [51]. Cellular stressors, including: serum deprivation, lysosomal inhibition and oxidative agents have been shown to lead to an increase in the release of alpha-synuclein [177]. These data also holds true in cell culture models; aggregates of alpha-synuclein have been found in the media of neuroblastoma cells as well as rat primary cortical neurons [144, 177]. We believe that gal-3 may be playing a role as we have data supporting the idea that gal-3 protein levels in neuronal cells decrease when exposed to aggregated alpha-synuclein. Here we are also able to provide live-cell movies.
Introduction

During times of cellular stress neurons have been shown to secrete alpha-synuclein [177]. It is unclear whether this is a protective mechanism, an attempt to purge the cell of a toxic protein. Interestingly, the alpha-synuclein recovered in the media demonstrates more oxidative modifications than other forms found intracellularly indicating a preferential release of damaged and aggregation prone alpha-synuclein [177]. Exosomes are small vesicles that break off from the endocytic machinery and manifest in the extracellular space. A membrane already envelops the contents of an exosome, which provides two benefits: ease of fusing with the plasma membrane of a recipient cell, and protection from enzymatic degradation in the extracellular space. The risk for extracellular degradation is apparent as unprotected alpha-synuclein has been shown to be degraded by matrix metalloproteinase 3 (MMP-3) in the extracellular space [178, 179]. Exosomes have been shown to be associated with secreted alpha-synuclein [121, 180, 181]. The exact form of alpha-synuclein may be irrelevant, as the secretion leads to the propagation of PD pathology. Uncontrolled expulsion of alpha-synuclein also may occur during cellular lysis from a dying cell.
It is well known that alpha-synuclein aggregates are toxic to cells, particularly in an in-vitro cell culture setting [182] and when neuronal cells are stressed they have been shown to release more alpha-synuclein [177]. We have been able to demonstrate that the short-term administration of alpha-synuclein aggregates to N27 and SH-SY5Y cells can lead to increased secretion of alpha-synuclein. The basal level of secretion for neuroblastoma cells and other cultured neurons has been described as a “regulated, low-probability event” [177]. Exposure of these neurons to alpha-synuclein aggregates for twenty-four hours leads to an increase in secretion into the cell culture medium as analyzed by western blot. Collectively, these experiments define a pathway by which pathology may be induced in cells during the spread of alpha-synuclein associated pathology observed in PD.

**Material and Methods**

**Cell Lines and reagents**

The human neuroblastoma cell line, SH-SY5Y, and the human monocyte cell line THP-1 were obtained from the American Type Culture Collection (ATCC). The rat dopaminergic neuronal cell line was a kind gift from Dr. Anumantha Kanthasamy [16]. SH-SY5Y cells were maintained and differentiated with 50 mM all L–trans Retinoic acid every 48–72 hours in Dulbecco's modified Eagle's medium (DMEM) supplemented with
10% fetal bovine serum (FBS)(Hyclone) and 100 IU/mL penicillin, 100 µg/mL streptomycin and 10 µg/mL ciprofloxacin. THP-1 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media, supplemented with 10% FBS, 100 IU/ml penicillin, 1 mg/ml streptomycin, 0.25 µg/ml amphotericin B, non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES buffer and 2 mM glutamine. The rat dopaminergic cell line N27 was cultured in RPMI media with the same additives used in the DMEM. Cells were maintained in a 37°C incubator with 5% CO2.

**Alpha-synuclein**

Full length alpha-synuclein and E46K mutant alpha-synuclein were purchased (rPeptide) and the lyophilized protein was rehydrated to a concentration of 1 mg/ml. alpha-synuclein was incubated for 3 days at 37°C in PBS with 100 mM NaCl under constant agitation followed by aliquoting and storage at −80°C. Aggregates were fluorescently labeled with Dylight 488 N-hydroxysuccinimide (NHS)-ester fluorophores (Thermo Scientific), according to the manufacturer's protocol prior to use.

**1-methyl-4-phenylpyridine (MPP+) Treatment**

MPP⁺ was obtained from Sigma-Aldrich in a powdered form. It was dissolved in DMSO and added to the RPMI cell culture media at a concentration of . This treatment lasted 24 hours after which the cells were washed briefly with PBS and new media added to the cell culture dish as well as “recipient” cells, N27chGal3. These “recipient” cells
were left in co-culture for 48 hours, at which time the media was removed and the cells were fixed and imaged.

**Western Blot of Supernatant**

N27 and SH-SY5Y cells were exposed to 24 hour of alpha-synuclein aggregate exposure followed by a complete media change for another 24 hours and then the cell culture supernatant was collected. 2× sample buffer was added to the supernatant and equal amount of protein was loaded into a 10% non-denaturing gel for electrophoresis. Proteins were transferred to nitrocellulose membranes and detected by incubation with the primary alpha-synuclein antibody. The H3C monoclonal alpha-synuclein antibody developed by Julia George was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Secondary antibody conjugated to HRP (Thermo Scientific) was used, and antibody complexes were detected using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific). Chemiluminescence was measured using a BioRad ChemiDoc XRS imaging system (BioRad)

**Immunofluorescence Microscopy**

Cells were allowed to adhere to Fibronectin (Sigma-Aldrich) treated glass coverslips (Fisher Scientific) and fixed with 3.7% formaldehyde (Polysciences) in 0.1 M
piperazine-N, N'bis(2-ethanesulfonic acid) PIPES buffer at pH 6.8 for 15 min. Cells were then permeabilized for 20 minutes in PBS with 10% Normal Donkey Serum (NDS) and 0.5% saponin (Sigma-Aldrich). Staining with specific monoclonal or polyclonal antibodies was done in 10% NDS with 0.5% saponin for 20 minutes. Mouse-anti alpha-synuclein (BD Biosciences) was immunostained at 1:400 dilution. Primary antibody was secondarily labeled with fluorophore-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch). Images were collected with a DeltaVision microscope (Applied Precision) equipped with a digital camera (CoolSNAP HQ; photometrics), using a 1.4-numerical aperture (NA) 100X objective lens, and were deconvolved with SoftWoRx deconvolution software (Applied Precision). Tiff images and 3-dimensional reconstructions were generated using Imaris software (Bitplane).

**Live Cell Imaging**

Images were acquired on a Deltavision deconvolution microscope equipped with a Weather Station™ chamber utilized to maintain the cells at 37° C at 5% CO2. Images were acquired using an Cascade 2 EMCCD (Photometrics) and deconvolved as described above.

**Image Analysis**

Deconvolved images were analyzed for quantification of Galectin-3 (Gal-3) signal by use of the Imaris software package (Bitplane).
Flow Cytometry

N27 and SH-SY5Y cells expressing mcherryGal-3 were plated in 6-well plates. N27 and SH-SY5Y cells expressing mcherryGal-3 and untagged GFP were also plated in 6 well plates. Alpha-synuclein aggregates were added to the cell culture media at a concentration of 3µg/ml for 24, 48 and 72 hour time points. Cell culture media was aspirated; cells were washed briefly with PBS, and then trypsinized. Cells were resuspended in the appropriate cell culture media and spun at 5,000 rpm for 5 minutes. Media was aspirated and cells were resuspended in PBS and Flow Fixative (50/50 volumetric mixture of PBS and 3.7% formaldehyde (Polysciences)). Cells were analyzed at the Loyola University Medical Center FACS Core Facility utilizing a LSRFortessa cell analyzer (BD Biosciences). For each sample in each experiment over 10,000 events were recorded and analyzed. All FACS analyses were performed using FlowJo (Treestar) software.

Statistical analysis

The data, wherever applicable, are presented as means + SEM and were analyzed using ANOVA. p<0.05 was considered statistically significant.

Results

Alpha-synuclein aggregate treatment induces cells to secrete more alpha-synuclein
To determine the effects of in-vitro aggregated alpha-synuclein on N27 and SH-SY5Y cells production of endogenous alpha-synuclein we measured via non-denaturing western blot the amount of protein in the supernatant. This western blot analysis took place after the cells had been treated for 24 hours with alpha-synuclein aggregates, washed and had the media replaced with untreated media for another 24 hour time span and then the supernatant was harvested and run on western. From Figure #19 it is clear that the addition of alpha-synuclein aggregates induces an increase in the amount of alpha-synuclein in the supernatant. Whether this is due to an increase in production of alpha-synuclein, a decrease in degradation or a fluctuation in the extrusion of alpha-synuclein into the supernatant is unclear.

**Figure #19.** Alpha-synuclein aggregate treatment induces an increase in endogenous alpha-synuclein secretion. N27 cell supernatant was run out on a non-denaturing gel, as was SH-SY5Y cell supernatant. Both cell lines were treated with 3µg/ml of wild-type alpha-synuclein aggregates for 24 hours, washed and media replaced. The new media was collected after a span of 24 hours and run in conjunction with the untreated supernatants. These blot images are representative of three independent experiments.
Cell to cell transfer of alpha-synuclein induces vesicular rupture

To determine whether alpha-synuclein expressed from one cell could induce the rupture of vesicles in neighboring cells, we treated N27 cells stably expressing alpha-synuclein (N27 alpha-synuclein) with the mitochondrial toxin MPP⁺ (1-methyl-4-phenylpyridine), which is used to induce a Parkinson's like pathology in tissue culture [183]. Following MPP⁺ treatment, we added N27chGal3 cells to the culture and incubated these cells together for a period of 48 hours. In this mixed culture, colocalization of alpha-synuclein and chGal3 could indeed be observed. In the example shown in Figure #20, alpha-synuclein can be observed localizing to crescent shaped structures at the periphery of chGal3 positive vesicles. We therefore conclude that alpha-synuclein expressed and released from eukaryotic cells can subsequently be internalized and induce vesicle rupture in a manner similar to what we observed with alpha-synuclein aggregates generated with recombinant protein.
**Figure #20:** Vesicle rupture following the cell to cell transfer of alpha-synuclein. N27 cells stably expressing alpha-synuclein and treated with MPP⁺ for 24 hours. N27chGal3 cells were then added to the culture and co-cultured for 48 hours. Cells were then fixed and stained for alpha-synuclein (green). The boxed areas in the left panel are enlarged to allow visualization of alpha-synuclein and chGal3 colocalization.

Galectin-3 transfer with labeled alpha-synuclein aggregates
While the experimental results shown in Figure #20 are compelling, we were very intrigued by the possibility of observing this translocation of alpha-synuclein in real-time. According to the Brundin group, the direct transfer of alpha-synuclein aggregates had never been observed [17]. We treated SH-SY5Y cells that were expressing mcherry tagged galectin-3 with 3 µg/ml of Dylight 488 labeled alpha-synuclein aggregates for 24 hours. After which the media was aspirated off, cells were washed and colorless media was added. Images were collected by an electron-multiplying charge coupled device (EMCCD) camera at five second intervals for 5 minutes. In Movie 1 the aggregates are in green and the gal-3 puncta are in red. There are certain cellular locations which show the colocalization of ruptured lysosomes, as evidenced by the red (gal-3) puncta, with labeled alpha-synuclein. Several of these intracellular protein associations appear to move quickly and fluidly from one cell to another.

**Movie #1. Demonstration of alpha-synuclein associating with Galectin-3 puncta and transitioning from once cell to another.** Live-cell movie of SH-SY5Y chGal3 cells that have been exposed to Dylight 488 conjugated alpha-synuclein aggregates for 24 hours. The chGal3 signal is red, and the labeled alpha-synuclein aggregates are displayed in green. Images were collected at five second intervals for five minutes.

*An unexpected decrease in mCherry-Gal-3 fluorescence*
While attempting to quantify the fluorescence of images taken in Figure #11 it was determined that the maximum fluorescence intensity of mCherry-Gal3 cells (N27 and SH-SY5Y) treated with alpha-synuclein aggregates had values in the same range as the same cells that were untreated (data not shown). This is a perplexing phenomenon as one of the tenets of fluorescent microscopy states that if the intracellular protein levels stay the same, then when the proteins aggregates they should exhibit a higher maximal intensity. This has held true for all previous studies in the lab, utilizing fluorescent microscopy [184, 185]. From this data we were interested in testing the intracellular levels of mCherry-Galectin-3 using flow cytometry before and after the addition of 3 µg/ml wild-type alpha-synuclein aggregates. Flow cytometry was chosen as the method of inquiry because it is able to provide analysis of large quantities of cells and we were only interested in the mean fluorescent intensity (MFI) of the mCherry-Galectin-3 channel. Cells were plated, aggregates were added for the specified time points, media was aspirated and cells washed with PBS and placed in flow fixative and run on a LSRFortessa (BD Biosciences). After three independent experiments where over ten thousand events were monitored for each experimental group we were finally able to deduce that following treatment with alpha-synuclein aggregates the MFI of intracellular mCherry-Galectin-3 was declining (Figure #21). This trend could be seen beginning after 24 hours of treatment with wild-type alpha-synuclein aggregates and continued, but does not appear to increase, up to 72 hours. Comparing each time point’s untreated group with the aggregate treated group gave us a significant (p>0.05) value. This data set
was very intriguing as, a paper had been recently published detailing the discovery that galectin-8 was playing a role in not only monitoring lysosomal integrity upon *Salmonella* invasion but when exposed to the intracellular environment galectin-8 activated antibacterial autophagy [54]. Galectin-8 and galectin-3 are both from the same family of cytosolic lectins, which led us to hypothesize that galectin-3 might have a dual function in the response to the lysosomes ruptured by alpha-synuclein. In fact, given the data we had collected in Figure #19 that alpha-synuclein was being vacated from the intracellular environment, we hypothesized that galectin-3 may be playing a role in the transfer of alpha-synuclein aggregates from cell-to-cell which was seen in *Movie 1*. 
Figure #21: Decrease seen in the MFI of neuronal cells chGal-3 after the addition of alpha-synuclein aggregates. N27 cells are seen beginning at 24 hours and continuing over the course of 72 hours maintaining a decrease in the MFI of mchGal-3 after treated with alpha-synuclein aggregates. Over 10,000 events were run for each sample and values are ± SEM. Data is representative of three independent experiments. *, p<0.05 as compared to N27 Gal3 untreated samples.

The role of galectin-3 in the secretion/transfer of alpha-synuclein

In order to test this hypothesis we created a stable dual fluorescent protein expressing cell line. N27 cells which expressed mCherry-Gal-3 and an untagged GFP
protein. These cells would be able to help determine whether galectin-3 was playing a specific role in the transfer/secrection of alpha-synuclein or it was merely subject to a diffusion gradient and effluxed from the intracellular environment due to the small pores that have been attributed to alpha-synucleins’ interaction with lipid membranes [15, 16, 18, 41, 42, 100, 105-107, 109, 115]. If the transport of alpha-synuclein was indeed being facilitated by galectin-3 then the data would show only a decrease in intracellular MFI values of mCherry-Gal-3 while the untagged GFP levels would stay the same. However, the elegant beauty of this experiment is that the data would also be able to demonstrate if The cells were plated and 3 µg/ml of alpha-synuclein aggregates were added. The media was aspirated, cells were washed with PBS and cells were collected at the specified time points (24, 48 and 72 hours) and placed in flow fixative and run on a LSRFortessa (BD Biosciences).
**Figure #22:** Intracellular decreases are seen in both mCherryGal-3 and untagged GFP after the administration of unlabeled wild-type alpha-synuclein aggregates. N27 cells exhibit a non-specific decrease in fluorescence after being exposed to alpha-synuclein aggregates. There is a decrease seen at all three time points (24, 48 and 72 hours) in both fluorescent channels (mCherry and GFP). Values are means ± SEM, from 3 independent experiments, with 10,000 events recorded per experiment. *, p<0.05 as compared to N27 Gal3 and N27 Gal3 + GFP untreated samples.

This data collected from the dual color expressing cells shows the same trend seen in **Figure #21**, the diminution of mCherry-Galectin-3 signal after 24 hours of exposure to
alpha-synuclein aggregates. This trend as seen in the previous figure continues for all 3 separate time points (Figure #22). However, the data provided due to the addition of the untagged-GFP clarifies the actions of both alpha-synuclein and galectin-3. GFP levels, while not as robust as mCherry-Gal-3 levels in the untreated samples, also decrease significantly upon the addition of wild-type alpha-synuclein aggregates. We took this as evidence that the addition of alpha-synuclein aggregates provided a non-specific method of efflux for intracellular proteins. As previously cited, alpha-synuclein has been known not only to bind and bend lipid membranes but also to create small pores in cellular membranes [15, 16, 18, 41, 42, 100, 105-107, 109, 115]. The creation of these pores is what we believe is leading to the efflux of mCherry-Gal3 as well as alpha-synuclein and this is evidenced by the non-specific decrease in GFP seen following the addition of alpha-synuclein aggregates.
CHAPTER SIX
SUMMARY AND DISCUSSION

Parkinson’s disease and Alpha-synuclein

Parkinson’s affects large numbers of people worldwide currently [5-7] and those numbers have been predicted to rise sharply in the coming years as large percentages of the population continue to age and life expectancy rates continue to increase. Genetic studies of small cohorts and families affected by early-onset and rapidly progressing forms of PD have led the biomedical focus to be placed on the protein, alpha-synuclein [34, 86, 115, 186, 187]. Neurodegeneration due to this nefarious protein is not limited to PD. Other neurological conditions have been found to have proteinaceous underpinnings of neurodegeneration due to alpha-synuclein and they include: PDD, DLB, and MSA. All of these diseases have been termed synucleinopathies or Lewy Body Diseases (LBD) as the common theme uniting all of these conditions is the presence of alpha-synuclein protein inclusions, called Lewy Bodies. The spread of these inclusions within the nervous system of patients has led to the proposal of the Braak hypothesis [117, 118, 188] in which a systematic propagation of the pathology is seen through progressive stages affecting different areas of the CNS. This viral-like property of the alpha-synuclein
protein is reminiscent of the pathology of adenovirus protein VI described in detail by the Wiethoff lab [22, 26, 27, 126-128]. Also, protein VI and alpha-synuclein share a remarkably similar structure. These two factors combined to give us the foundation for our study: that alpha-synuclein due to its similar structure to adenovirus protein VI would have a similar membrane-rupturing function and closely-related downstream consequences of this rupture.

Summary of Results
In the previously described experiments, we tested the hypothesis that alpha-synuclein aggregates induce the rupture of lysosomes. This hypothesis was founded on the observation that alpha-synuclein and adenovirus protein VI can both induce the tubulation and rupture of synthetic membranes [22, 42, 126, 127]. Given that protein VI is the viral protein responsible for inducing vesicle rupture during infection [22, 26, 27, 126-128], we hypothesized that alpha-synuclein may possess a similar activity that is relevant to its pathology. Utilizing the relocalization of Galectin-3 (Figure 2) as a marker of vesicular rupture [54, 138-141, 151], we demonstrate that alpha-synuclein aggregates can induce the rupture of vesicles in two neuronal cell lines: N27 and SH-SY5Y. We demonstrate that the ruptured vesicles induced by alpha-synuclein are positive for LAMP2 (Figure 4), and that the appearance of these ruptured vesicles correlates with the induction of a cathepsin B dependent increase in ROS in these cells (Figure 7).
The Wiethoff lab has previously shown that release of protein VI correlates with the rupture of cell membranes prior to, or upon reaching early endosomes [27, 127]. Early escape from endosomes may be favorable for human adenovirus 5, as it results in the release of fewer activated cathepsins into the cytosol and a reduced inflammatory response compared to other human adenovirus serotypes that enter cells by using different receptors [127]. Conversely, vesicle rupture by alpha-synuclein is likely not a property that provides selective advantage to the organism. Rather, it seems likely that this property may be an indirect and evolutionarily unintended activity of a protein that has likely evolved to perform a function other than vesicle rupture. Although it is possible that the alpha-synuclein aggregates used in our study do not precisely recapitulate the ability of toxic alpha-synuclein species to mediate vesicle rupture in PD, a number of other in vitro and in vivo studies collectively suggest lysosomal dysfunction may be a central aspect of PD pathology (reviewed in [94]).

The Lansbury group has demonstrated that alpha-synuclein can induce both small membrane pores on artificial membranes, which allows for the diffusion of ions and very small molecules, as well as a more dramatic permeabilization at higher concentrations, allowing larger molecules and proteins to pass through the lipid bilayer [15, 189, 190]. Although our studies do not directly compare these two possibilities, the molecular weight of the mCherry-Gal3 fusion protein is much larger than the size limit of pore
diffusion reported in these studies, suggesting that membrane rupture, rather than pore formation, is required for the localization of chGal3 to vesicles ruptured by alpha-synuclein.

Biochemical analysis of the alpha-synuclein aggregates utilized in this study reveals the presence of both fibrillar and oliomeric species of alpha-synuclein (Figure 1). Future studies will be required to determine which forms of alpha-synuclein mediate vesicular rupture most efficiently. The aggregation prone alpha-synuclein variant E46K, which tended to form aggregates too large to enter the endocytic compartment (Figure 8), did not induce ruptured vesicles to the same degree as wt alpha-synuclein, nor did it induce ROS levels equivalent to wt alpha-synuclein (Figure 9). This does not demonstrate that E46K alpha-synuclein has less pathological potential than wt alpha-synuclein. In fact, the E46K mutation is linked to familial PD [86]. However, in the context of these experiments, we suspect that the tendency to self-associate in vivo into pathological species of alpha-synuclein is mimicked by the protocols employed here to induce the aggregatation of wt alpha-synuclein. When the E46K alpha-synuclein mutant was subjected to similar protocols, much larger species of aggregates were induced which allowed us to compare the relative pathological potential of large aggregates and small aggregates in our experimental system. This is consistent with a previous report suggesting that E46K has an increased propensity to assemble into larger, insoluble fibrils with an amyloid architecture [191]. We cannot exclude the possibility that the
reduced vesicular rupture and ROS induction by the E46K mutant is due to the E46K mutation. However, we favor the idea that these larger aggregates induced with the E46K variant were less toxic because they were too large to be internalized into target cells, preventing them from inducing the rupture of intracellular vesicles.

The fact that chGal3 positive ruptured vesicles do not ubiquitously contain detectable amounts of alpha-synuclein suggests that alpha-synuclein has the capacity to dissociate from the endocytic vesicle following rupture, although we cannot exclude the possibility that these vesicles contained alpha-synuclein which was not detected in our experiments. Similarly, not all alpha-synuclein signal we observed colocalized with chGal3. This may indicate alpha-synuclein which has dissociated from a vesicle following rupture or alternatively may be alpha-synuclein still existing within the vesicular compartment which has not induced vesicle rupture. While we cannot distinguish between these possibilities using this assay, the observation that alpha-synuclein localizes to the periphery of ruptured vesicles is consistent with the idea that alpha-synuclein induces membrane curvature capable of inducing the rupture of these vesicles.

It is also worth noting how the pathological pathway identified here might be relevant to the propagation of alpha-synuclein pathology in vivo. Work from other labs, taken with the data presented here, suggests a mechanism by which alpha-synuclein
mediated lysosome rupture may perpetuate the propagation of alpha-synuclein pathology. Specifically, Alvarez-Erviti and coworkers have demonstrated that lysosomal dysfunction and stress increase the release of alpha-synuclein containing exosomes [180]. Danzer and colleagues have demonstrated alpha-synuclein containing exosomes induce more pathology than recombinant aggregates [192]. Taken together, the observations here and in these studies suggest a mechanism by which continued vesicular rupture by alpha-synuclein may not only exert toxic effects on a given cell but may also perpetuate the propagation of alpha-synuclein pathology to neighboring cells.

Although the data presented here utilize cell lines to demonstrate lysosomal rupture and ROS induction, it is worth nothing that others have reported clinical observations consistent with the pathway identified. Reduced cathepsin and LAMP immunoreactivity has been observed in nigral neurons in PD patients [153], consistent with the idea of vesicle rupture and cytoplasmic diffusion of lysosomal contents. The role of mitochondrial dysfunction is also well established in PD [148].

Here, we define the pathway by which alpha-synuclein escapes the vesicular compartment and induces toxicity in tissue culture cells. Future studies are needed to determine the degree to which α-synuclein mediated lysosomal rupture affects the
propagation of PD pathology in primary neuronal cultures, animal models and individuals affected by PD and other synucleinopathies.

**Figure #23.** Schematic of molecular mechanism for alpha-synuclein pathology.
Future Directions

While the data presented here is compelling, we recognize that it was obtained through the use of cultured cells. Cultured cells provide an expedient method to investigate molecular mechanisms of pathology. Albeit, an occasionally unreliable one. The future of this project is defined by several broad aims. Foremost of these aims will be the replication of this or similar experiments in another model. Primary murine neuronal culture has been recently established as a new standard in the veracity and applicability of results from cultured cells. This type of cell culture method has been a staple of the Surmeier and Lee labs and has been utilized in a number of key findings in high impact journals [13, 57, 69, 71, 150, 173, 193]. Another model that would provide great insight into the molecular mechanisms that we have detailed here would be the single inoculation of alpha-synuclein aggregates into the striatum of wild-type nontransgenic mice as detailed by Luk and colleagues in a 2012 paper published in Science [123]. These studies would be done with the ultimate goal being to investigate age-matched PD and control human specimens for the presence of galectin-3. A second broad aim which the genesis of has been this current study is to analyze the efficacy of cathepsin-B inhibitors. The data presented in Figure #16 demonstrates that aggregated alpha-synuclein leads to the generation of high levels of ROS by neuronal cells and that these high levels of ROS can be almost completely ameliorated by the addition of the cathepsin-B inhibitor, Ca-074me. A variety of cathepsin inhibitors have been explored for efficacy in tumor survival and
metastasis [194-197]. Yet, there seems to be a relatively large knowledge gap concerning cathepsins and neurodegenerative disease. Cathepsin D has been shown to be upregulated in the caudate nucleus [198], and to affect alpha-synuclein processing [199]. An Italian group has very recently shown that the addition of Ca-074me in their hands also leads to an amelioration in ROS production as well as Interleukin-1 beta (IL-1β) [200]. These studies were performed on human monocytes yet the similarities in experimental design and data speak to the veracity of the finding. Cathepsin inhibition could also be a straightforward way to halt further downstream consequences and hopefully neuronal cell death in PD patients. The final broad aim that would be very helpful to elucidate, given the findings stated here, is to determine the role that endogenous galectin-3 plays in the pathology of PD. Galectin-3 has been detected intracellularly and extracellularly in a variety of tissues [201]. Expression of galectin-3 in the CNS has not been extensively investigated, but microglia have been shown to express galectin-3 [202, 203]. Neuronal expression remains to be seen. However, this provides an interesting possibility as galectin-3 has been shown to be a marker for intracellular vesicle rupture. Alterations in the expression level of galectin-3 may be one of the key missing pieces to the identification of patient populations at-risk for PD. Modulation of galectin levels may provide the cell with a way to secrete/escape the pathology of aggregated alpha-synuclein. It is these several broad aims which represent the next series of questions needed to move this project forward.
Clinical Implications

The data presented herein, are applicable to variety of clinical settings and offer novel information regarding alpha-synuclein and galectin-3 as a potential biomarker and arbiter of downstream consequences leading to neurodegeneration. Our data, demonstrating galectin-3 as a marker for lysosomal rupture in neuronal cells and an increase in cathepsin-B dependent ROS following alpha-synuclein aggregate treatment implicates cathepsin-B as a possible therapeutic target in the treatment of Parkinson’s related pathology. In addition, reliance on galectin-3 could be a beneficial biomarker for the diagnosis and prognosis of PD and other synucleinopathies.
REFERENCES


VITA

David Freeman was born and raised in Elmhurst, Illinois. He attended Augustana College and earned a Bachelor of Arts in Art History and Biology. Following his undergraduate education he attended Loyola University Chicago for graduate school in the neuroscience program under the advisorship of Fletcher White M.S., Ph.D. After receiving his Masters of Science degree he began the M.D., Ph.D. training program at Loyola University Chicago – Stritch School of Medicine. During that portion of his education he spent his graduate school training in the lab of Edward Campbell Ph.D. Upon successful completion of both degrees he began his neurosurgical training at the University of Minnesota.